HÆMATOLOGICAL TECHNIQUE

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BY

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THIRD EDITION

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PREFACF TO THIRD EDITION

Dr L E Napier left India early in 1943 As suggested by him I have to take up the responsibility of bringing out the third edition

The second edition was exhausted as rapidly as the first edition and the book was out of print for over a year as it had not been possible to bring out this edition earlier due to war time difficulties

In the new edition the section on the development of red cells has been re written following the Schemata outlined by Israels (1941) The chapters on blood grouping and blood transfusion have been enlarged In the table of contents sub headings have been given under each main heading

I am grateful to Dr J B Chatterji M B Assistant research worker Hæmatological Unit I R F A for assistance with the proofs

School of Tropical Medicine
Calcutta
The 13th August 1945

C R D G

PREFACE TO SECOND EDITION

The rapid exhaustion of the first edition of this book indicates that there is a demand in India for a book of its kind

In this second edition we have included blood grouping and transfusion Though neither is a diagnostic method we have felt that these two chapters would not be out of place in this book and with the closer approach of the war to India there is a special demand for knowledge on these subjects

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PREFACE TO FIRST EDITION

In the past the attitude of the medical profession in India towards anæmia has been that it was an almost inevitable accompaniment of residence in a tropical country and that therefore *per se* it need not claim attention It is now beginning to be realized that when a patient is anæmic there is always some pathological process to account for the fact and that whilst other methods of ascertaining the nature of these pathological processes should not be neglected much information

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may be obtained from the blood picture Consequently far more attention is being paid to anæmia itself and the importance of accurate methods of blood examination for the proper study of a case of anæmia is gradually being appreciated

Many books on laboratory technique give full details of all the various methods that can be adopted but such books are not always within the reach of practitioners further the methods described in these books are often embarrassing in their multiplicity We therefore felt that readers of the *Indian Medical Gazette* particularly those working in India might find a short description of the methods followed at the Calcutta School of Tropical Medicine of some practical use and have prepared a series of short notes on hæmatological technique these were published in the *Indian Medical Gazette* during 1940 and early 1941 We are now issuing them as a collected series of papers

In a few instances original modifications have been introduced but for the most part the methods we have described have been thoroughly tested by laboratory workers in many parts of the world and are generally accepted as sound and accurate in every instance they are methods that we have used in our laboratory for some considerable time in most instances for many years and found not only accurate but suitable for the local conditions

We have limited these articles to the description of procedures that are more-or less routine in the investigation of a case of anæmia in the Calcutta School of Tropical Medicine but we have not attempted to be comprehensive for in the majority of our cases in order to trace the ætiological factors many other examinations protozoological bacteriological helminthological and serological and in addition certain other biochemical examinations such as the estimation of cholesterol blood protein blood calcium etc have been carried out usually in other departments of the School We also group the blood in every case on admission but as this is not in any sense a diagnostic procedure we have not included it in this series

At the time we commenced this series all the apparatus we described was available in India we regret that now some of it is not but we hope that reliable British or American substitutes for the apparatus of German origin will soon be available

Our thanks are due to Dr P C Sen Gupta M B (Cal) for allowing us to include the article on Red cell diameters Price Jones curve of which he was the joint author in this volume

School of Tropical Medicine
Calcutta
June 19th 1941

L E N

HÆMATOLOGICAL TECHNIQUE

1

THE COLLECTION OF THE BLOOD SAMPLE

THE first essential is that one constant practice should be decided upon and followed There is a difference between capillary which for all practical purposes is arterial and venous blood but the difference is only of practical importance if two samples from the same individuals at different times are to be compared or when normal standards are being worked out

As there are very distinct advantages in the use of venous rather than capillary blood the former is preferred

The advantages are that all the examinations e g the estimation of hæmoglobin the determination of cell volume the total red and white cell count the reticulocyte count the differential white cell count the measuring of the red cell diameters the icterus index the van den Bergh reaction the fragility test the erythrocyte sedimentation test etc can be done from a single sample of blood obtained by a single puncture Further the tests can be repeated by the same or by different workers from the same sample of blood

For some of the tests the van den Bergh test for example blood will have to be taken from the vein so that sufficient might as well be taken at one time for all the tests and the patient saved a number of additional pricks In weak and anæmic individuals it is sometimes difficult to draw enough blood from a finger or ear lobe without squeezing the part and thereby diluting the blood with the tissue fluid

The following rules should be observed when using venous blood —

(a) The syringe should be air tight and perfectly dry The syringe is conveniently dried by first washing it thoroughly in clean water removing the water with alcohol removing the alcohol with ether and finally drawing air through it If there is even a trace of water or alcohol there will be hæmolysis of the blood and this will make it unsuitable for examination

(b) The tourniquet should not be kept tied for more than 2 minutes when drawing the blood from the vein as the red cell count begins to increase after 3 minutes stasis

(c) The needle of the syringe should be removed before the blood is put into the flask as hæmolysis may result if the blood is forced through the needle

Icterus index and van den Bergh test—2 hours
Cell volume fragility test and sedimentation rate—3 hours
Hæmoglobin estimation red cell count white cell count and reticulocyte count—24 hours

2
ESTIMATION OF HÆMOGLOBIN

Hæmoglobin is a conjugated protein consisting of a protein globin and an iron containing pigment

Hæmoglobin readily combines with oxygen to form oxyhæmoglobin each molecule of hæmoglobin contains one atom of iron which is capable of combining with two atoms of oxygen

The important functions of hæmoglobin are to convey oxygen to the tissues to remove carbon dioxide from the tissues and in maintaining a constant reaction of the blood

To estimate the amount of hæmoglobin in any sample of blood advantage is taken of certain properties of hæmoglobin Of these the most important is its oxygen carrying capacity A fixed amount of hæmoglobin will always combine with the same amount of oxygen actually 1 gramme of hæmoglobin with 1 34 c cm of oxygen the amount of oxygen that will combine with a sample of blood can be measured accurately and the hæmoglobin content of the sample thus estimated

Another property is its iron content Hæmoglobin has a known molecular formula in which there is one atom of iron so that by estimating the amount of iron in red cells from a given volume of blood it is possible to calculate the amount of hæmoglobin in the sample

Hæmoglobin is a protein with a known refractometric index By measuring this index it i possible to calculate the amount of hæmoglobin in any sample

Yet another property of hæmoglobin is its colour and this can be measured in the various ways indicated below

Whilst the property of hæmoglobin easiest to measure is its colour the most fundamentally important property is its oxygen carrying capacity There is evidence that these two properties are very closely correlated so that if an instrument which measures the colour is first calibrated by the oxygen carrying method a very close approximation of the hæmoglobin content of a sample can be obtained by the former method

Most clinical hæmoglobinometers that are now sold are thus calibrated

Clinical methods of estimating hæmoglobin—Of the many methods of estimating hæmoglobin that are in use none of the simpler methods is above criticism while the more accurate procedures e g van Slyke s oxygen-carrying capacity method are hardly suitable for general clinical practice Most of the clinical procedures in common use are methods of measuring the colour of the hæmoglobin and are based on one or other of the following principles —

(d) The flask should always be kept closed with a rubber cork cotton wool should never be used The flask should be shaken at once vigorously to ensure complete solution of the oxalate powder
(e) When not in use the flask must be kept corked and in a cool receptacle during the summer
(f) The blood should always be mixed thoroughly for at least 3 minutes preferably in a shaking machine before any sample is withdrawn from it The blood should always be taken directly from the flask and not from blood poured out on a slide or watch glass

Time for collection of blood —When repeated examination is required blood should always be collected at the same hour under basal conditions in the morning Even for a single examination it is preferable to collect the blood in the morning under basal conditions this precaution will minimize the effect of the fluctuations in the total and differential count due to muscular and other physiological activities

Procedure —The blood is drawn from one of the prominent superficial veins at the bend of the elbow in a perfectly dry air tight syringe

First of all without detaching the needle small drops of blood are put on clean glass slides and smears for the differential leucocyte count and red cell diameter measurements should be made* then the needle is detached and a measured amount of blood is transferred to a small 25 c cm Erlenmeyer flask containing the requisite amount of anticoagulant for the quantity of blood to be taken

Anti coagulant —Two milligrammes of dry oxalate powder is required for each cubic centimetre of blood A mixture of potassium and ammonium oxalate powder in the proportion of 2 to 3 has been found to be ideal as in the above proportion the shrinkage caused by potassium oxalate is counteracted by ammonium oxalate (Wintrobe and Landsberg 1935) To measure the requisite amount of the oxalate powders make 1 per cent solution of the two oxalate powders ammonium and potassium accurately measure with a pipette 0 4 c cm of the potassium salt and 0 6 c cm of the ammonium salt and put them into the flask evaporate in a dry oven after which the oxalate will be found at the bottom of the flask in a powdered state this is the amount required for 5 c cm of blood for 3 c cm the proportions will be 0 24 c cm potassium and 0 36 c cm ammonium salt The flasks are now kept corked and are ready for use

It is a good practice to have two sets of flasks prepared containing enough anti coagulant for 5 c cm and 3 c cm respectively

If potassium oxalate alone is used 2 milligrammes are required for each cubic centimetre of blood that is 10 milligrammes or 1 c cm of 1 per cent solution for each 5 c cm of blood and in this case Wintrobe s correcting factor × 1 09 must be applied to compensate for shrinkage

Time limits —Osgood and others (1931) give the following time limits for different examinations —

Making smears for differential count—1 hour

* As stated above this can be done from the oxalated sample but it is better to make separate smears

writers vary from 13 8 to 17 3 grammes and in our personal experience we have found amongst groups of so called healthy coolies in Assam a figure lower than the former and amongst healthy Europeans in India one higher than the latter If the expression 100 per cent hæmoglobin has any meaning at all it must imply that the particular sample is what one would expect from that individual if he were in perfect health This would mean that a different standard would have to be adopted for each class of individual a procedure which would lead to endless confusion It is therefore better to abandon the second method namely that of giving the hæmoglobin in terms of a percentage of an unknown and arbitrary standard and to adopt the first and express results in terms of grammes of dry hæmoglobin per 100 c cm of blood

Instrument makers who have hitherto made no attempt to adopt a universal standard and hæmatologists who have not encouraged them to do so are both responsible for the present unsatisfactory state of affairs

The confusion that exists cannot be better exemplified than by the table (I) below which shows that not only are there differences in the standard between instruments of various makes but instruments of the same name made at different times vary from one another and finally various authorities give different figures for apparently the same instruments

TABLE I

Amounts of hæmoglobin per 100 c cm of blood which are shown as 100 per cent by different instruments

Authority	Tallqvist	Sahli	Dare	Newcomer	Haldane	Fleischl Miescher
Lepper and Lartly 1933	13 8	17 3	13 77 (old) 16 9 (new)		13 8	15 8
Nicholson 1934	15 8	17 2 (old) 4 5 (new)	13 8	16 9		
Whitby and Britton 1939	13 8	17 3 (old)	15 9		13 8	
Krick and Carver 1937	15 8	17 3 (old) 13 8 (new)	13 77	16 9		
Levinson and MacFate 1937	15 8	17 3	16 0	15 92		
Ordway Gorham and Isaac 1937	15 8	13 8* to 17 2	13 7 to 16 0	16 9	13 8	15 8
Beck 1938	13 8	13 8 to 17 3	13 7 to 16 0	16 9		

* Different instruments are supplied with different standards

I Direct comparison of the colour of undiluted blood against a graduated colour standard

(a) On special blotting paper (Tallqvist)
(b) Between glass plates (Dare)

II Comparison of blood diluted to a fixed percentage against graduated colour standard

(a) Diluted with sodium carbonate solution and compared with a coloured glass wedge (Fleischl Miescher)
(b) Diluted with deci normal hydrochloric acid and compared with coloured glass wedge (Hellige Neoplan)

III Comparison of blood diluted to a varying degree with a fixed colour standard

(a) Diluted with water and compared with a permanent picrocarmine standard (Gowers)
(b) Brought into contact with carbon monoxide diluted with water and compared with a permanent colour standard (Haldane)
(c) Diluted with deci normal hydrochloric acid and compared with a permanent standard of acid hæmatin (Sahli) or a coloured glass block (Hellige)

IV Comparison of blood diluted with deci normal hydrochloric acid to a fixed percentage against a fixed colour standard in a colorimeter of the Duboscq type

(a) Water placed in one chamber of the colorimeter and a standard coloured disc interposed (Newcomer)

Other methods which require more elaborate instruments depend on the intensity rather than the colour of the light transmitted through hæmoglobin solutions this is measured by means of a photo electric cell

In the writers laboratory the new Hellige normal hæmometer is used for all clinical purposes The great advantage in the Hellige instrument is that it is possible to match the brown coloured acidulated blood solution with the colour standard exactly and as the standard is made of coloured glass it is unlikely to undergo any colour change one coloured prism which has been in use in our laboratory for more than six years has not undergone any colour change during this period Error excluding those of carelessness in technique using dirty pipettes etc may *arise through variations in the diluting pipettes and in the calibres of* the graduated tubes The method suffers the disadvantage that with each dilution only one reading can be made—a second observer can check the reading but not make an independent one—and that a time interval of 15 to 20 minutes must be allowed before reading the result

Methods of expressing the results —The amount of hæmoglobin in an individual s blood may be expressed—

(i) As the number of grammes per 100 c cm of blood or (ii) as the percentage of the amount present in the blood of the normal individual

The disadvantage in the latter method is that there is no uniformity of opinion as to what is a normal individual Figures for the normal given by different

writers vary from 13.8 to 17.3 grammes and in our personal experience we have found amongst groups of so called healthy coolies in Assam a figure lower than the former and amongst healthy Europeans in India one higher than the latter. If the expression 100 per cent hæmoglobin has any meaning at all it must imply that the particular sample is what one would expect from that individual if he were in perfect health. This would mean that a different standard would have to be adopted for each class of individual, a procedure which would lead to endless confusion. It is therefore better to abandon the second method, namely that of giving the hæmoglobin in terms of a percentage of an unknown and arbitrary standard, and to adopt the first and express results in terms of grammes of dry hæmoglobin per 100 c cm of blood.

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Pijper and Fairley 1933	13.8	17.3	13.77 (old) 16.9 (new)		13.8	15.8
Nicholson 1934	15.8	17.2 (old) 14.5 (new)	13.8	16.3		
Whitby and Britton 1939	13.8	17.3 (old)	15.9		13.8	
Krack and Carver 1937	15.8	17.3 (old) 13.8 (new)	13.77	16.9		
Leiman and McTate 1937	1[illegible].8	17.3	16.0	15.92		
Ordway, Gorham and Isaacs 1937	15.8	13.8 to 17.2	13.7 to 16.0	16.0	13.8	15.8
Beck 1938	13.8	13.8 to 17.3	13.7 to 16.0	16.9		

Different instruments are supplied with different standards

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TABLE II

Normal hæmoglobin levels of different populations

Sex	Age	Locality	Economic status	Hæmoglobin in grammes per 100 c cm	Standard deviation	Number on which based	Authority
Men	19—30	Bombay	Students	15.37	± 0.96	121	Sokhey *et al* 1937
	25—45	Calcutta	Mixed servants	14.77	± 1.36	50	Napier and Das Gupta 1935*a*
	25—45		Clerks and doctors	15.70	± 0.91	30	1936
	Adults	Assam	Coolies	12.63	± 1.41	[illegible]	1936
		Cachar		12.60	± 1.83	25	Napier and Majumdar 1938
		Assam		11.83	± 1.67	24	Napier and Das Gupta 1935*b*
		Shivrajpur		13.74	± 1.79	47	Sen (Napier 1939)
				12.[illegible]5	± 1.72	49	( )
		U S A		16.00			Castle and Minot 1936
		Britain		15.60			Whitby and Britton 1939
				14.50			Price-Jones 1931
Women	18—30	Bombay		12.99	± 1.10	101	Sokhey *et al* 1938
	14—38	Calcutta	Middle class	12.63	± 1.01	128	Napier Edwards and Das Gupta 1941
	17—2[illegible]	Madras	Students	13.73	± 0.93	62	Sankaran and Rajagopal 1938
	17—30	Delhi	Middle class	13.11	± 0.81	100	Benjamin 1939
		Coonoor (6 000 feet)		15.81	± 2.54	100	Radhakrishna Rao 1938
	Child bearing	Cachar	Coolies	10.40	± 1.74	25	Napier and Majumdar 1938
		Assam		10.80	± 2.30	20	Napier and Bilimoria 1937
		Britain		13.60			Price Jones 1931
				13.70			Whitby and Britton 1939
	18—22	Michigan	Students	13.76		50	Bethel 1936
Pregnant women		Assam	Coolies	10.[illegible]0	± 1.60	40	Napier and Bilimoria 1937
				9.22		228	Napier and Das Gupta 1937*b*
			(Obvious anæmics excluded)	[illegible]9	± 1.[illegible]2	192	1937*b*
		Michigan		11.85		8	Bethel 1936
		Coonoor (6 000 feet)	Ante natal clinic (mixed)	15.52	± 2.52	100	Radhakrishna Rao 1938

However many instruments that are now sold give the readings in terms of grammes of hæmoglobin per 100 c cm of blood others give readings in duplicate so that it is easy to see what corresponds to 100 per cent or if they use the older method they state what their 100 per cent corresponds to in terms of dry hæmoglobin

The excuse given for adhering to the older method (ii) is that most practitioners are familiar with this method of expressing the hæmoglobin It may be true that they are familiar with the expression say hæmoglobin 80 per cent but they don t know what it means for, it may mean anything between 11 and 13 8 grammes of hæmoglobin per 100 c cm of blood a discrepancy which is far from negligible They must therefore learn something new so why should they not learn a new method which has a definite meaning accepted by all hæmatologists Most American medical books are now using the new momenclature and in Great Britain even the more conservative clinicians will follow the lead already given by British hæmatologists in course of time

A table (II) showing the normal hæmoglobin levels in different population in India with a few samples from other countries for comparison is given

Apparatus required

(i) Hellige normal hæmometer with extra mixing tubes and pipettes
(ii) Rack
(iii) N/10 Hydrochloric acid
(iv) Distilled water
(v) Pipettes with rubber teats
(vi) Hand lens

Hellige normal hæmometer this consists of the following parts —

(i) A mixing tube with graduations from 10 to 170 (see figure 1 A)
(ii) A pipette with a mark at 20 c mm capacity (B)
(iii) A solid glass rod for stirring the mixture in the tube to ensure thorough mixing
(iv) Coloured prism or prisms against which the blood has to be matched (C)
(v) The housing of the hæmometer (D) which is made of steel with a large base to ensure safe standing Inside the housing are the coloured prisms in the front and an opaque glass plate at the back Through a hole in the housing the mixing tube is introduced and lies at the same distance from the eye as the prism

Technique —Fill the graduated measuring tube up to mark 10 (using a large pipette) with N/10 hydrochloric acid

After shaking the blood in the flask for 3 minutes with the pipette draw up the blood to the 20 c mm mark exactly wipe away any blood adhering to the outside of the pipette and transfer the blood into the measuring tube If the blood goes a little beyond the mark it is brought back to the mark by touching one s finger with the tip of the pipette a few times cotton wool or blotting paper should never be used

by using the conversion table of which the skeleton is given (Table III) It is recommended that the full table be prepared and kept handy

TABLE III

Skeleton table of hæmoglobin values of the Hellige normal hæmometer converted into grammes of hæmoglobin per 100 c cm of blood

Per cent Hellige	Grammes per 100 c cm	Per cent Hellige	Grammes per 100 c cm
1	0 1375	10	1 375
2	0 2750	20	2 750
3	0 4125	30	4 125
4	0 5500	40	5 500
5	0 6875	50	6 875
6	0 8250	60	8 250
7	0 9625	70	9 625
8	1 1000	80	11 000
9	1 2375	90	12 375

100 per cent Hellige = 13 75 grammes per 100 c cm

3
ENUMERATION OF ERYTHROCYTES AND LEUCOCYTES

Erythrocyte and leucocyte counts are expressed as the number of cells per cubic millimetre (c mm) of blood

Principle —In enumerating the red or white cells oxalated venous blood from the flask (or capillary blood from the finger or ear lobe) is taken into a red or white cell pipette up to a certain mark and the pipette is filled with the diluting fluid it is then shaken and a drop of the mixture is put into a special counting chamber where the cells within some specified ruled area are counted and finally the total number of cells per c mm is calculated

The dexterity necessary to carry out the various manipulations in the different stages of making a blood count can only be acquired by practice Further

By repeated filling and emptying the pipette should be completely freed from all vestiges of blood and the blood should be intimately mixed with the hydrochloric acid the red hæmoglobin now turns to acid hæmatin (brown)

Wait for 15 to 20 minutes the mixture which is now brown should be perfectly clear

Now add water slowly drop by drop with a pipette mixing constantly with the solid glass rod until the mixture matches exactly the colour of the standard prism C in the housing During the process the solid rod should not be placed on the table as each time it is allowed to touch anything the small amount of mixture adhering to the rod will be lost and this source of error will be multiplied

To match the solution and the standard the hæmometer should be held up to a good source of indirect natural light direct sunlight and artificial light being avoided The level in the mixing tube opposite the lowest point of the meniscus of the diluted acid hæmatin is read this gives the percentage of hæmoglobin The end point is generally very sharp and there is seldom any difficulty in matching the solution and standards exactly except with leukæmic and jaundiced blood When a point is reached at which the solution appears to match the standards take a reading then add a drop of water mix the solution hold up to the light and take a second reading If at the second reading the solution is definitely too light the first reading should be taken On the other hand if the second reading still appears to match the standards but after yet another drop has been added the solution is definitely lighter than the standards the result should be the mean of the first two readings

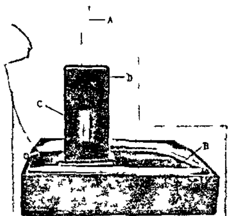


Fig 1 —Hellig normal hæmometer

The writers Napier and Das Gupta (1935) calculated that 100 per cent with the Hellige normal hæmometer represented 13 67 grammes by the refractometer method but later by means of the van Slyke oxygen carrying method a slightly higher figure was obtained and they decided to consider 100 per cent as 13 75 grammes

Other instruments of this pattern (Hellige normal hæmometer) that the writers have used have not differed appreciably from this standard but it is advisable to have the colour standard with the pipettes and mixing tubes to be used tested in some laboratory where the van Slyke oxygen carrying method or the iron estimation method is also employed

The reading in terms of percentage from the tube must be converted into grammes and the result expressed in grammes this can be done conveniently

platforms Upon each ruled area there are nine large squares one square millimetre each separated by double lines

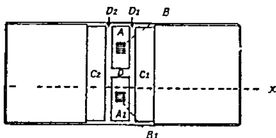


FIG 2 —Improved counting chamber

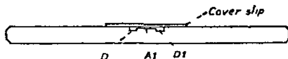


FIG 3 —Section at x of figure 2

The four large corner squares A B C and D are the areas in which the leucocyte count is made (figure 4) Each of these 4 square millimetres is subdivided into 16 squares to facilitate counting

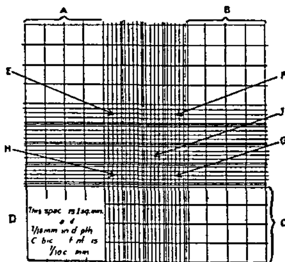


FIG 4 —Neubauer ruling

The square millimetre in the centre of the ruled area is used for the red cell count It is subdivided into 400 smaller squares each of which is therefore $\frac{1}{400}$ of a square millimetre

close attention must be paid to every detail of the technique if accurate results are to be obtained

Apparatus required

(i) Microscope
(ii) Hæmocytometer with counting chamber preferably with Neubauer ruling
(iii) Red and white cell pipettes
(iv) Hæmocytometer coverslip
(v) Diluting fluids
(vi) Watch glass

The ideal would be to use certified counting chambers and pipettes but where this is not possible on account of their high cost only those which are manufactured by reliable firms (viz Carl Zeiss Bausch and Lomb etc) should be used and these should at least be checked against certified instruments

Counting chambers—There are various types of counting chambers in use The new types of chamber have two main advantages over the old Thoma chamber —

(1) The coverslip can be placed in position upon its base before the blood is introduced and this may be done slowly and with the care that is essential to ensure the appearance and persistence of Newton s colour bands at the surface in contact

(2) The uneven distribution of the corpuscles on the counting surface which is liable to arise in the original chamber devised by Thoma and is due to the rapidity with which the corpuscles especially the red ones tend to settle in the diluent is obviated the new chambers are so devised that the diluted blood rapidly enters the counting space by capillary attraction

In the new types of chamber e g Neubauer Burker etc the counting space is oblong and divided into two compartments by a transverse groove both *of which contain a ruled area for counting the red as well as the white corpuscles* These two counting areas afford a means of making duplicate counts with a single application of the coverslip

In our laboratory we have used chambers with Thoma Burker and Neubauer rulings but for over eight years we have been using only chambers with the Neubauer ruling which is simpler to use than the Burker *with a single application* of the overslip a larger area for the leucocyte count 4 square millimetres on each side of the chamber as opposed to 1 square millimetre in the Thoma type is obtained

Counting chamber with improved Neubauer ruling

The different parts of the counting chamber are shown in figures 2 and 3

The middle platform A A with the ruled areas B B are exactly 0 1 mm *lower than the two side platforms* The middle platforms are separated from each other by the transverse groove D and from the two side platforms by the *trenches D and D When the coverslip is placed* upon the platform there is a space exactly 0 1 millimetre deep between it and the ruled areas on the middle

Diluting fluids —For the *red cell count* we have found the following solution the most satisfactory —

Sodium sulphate	12 5 grammes
Glacial acetic acid	33 3 c cm
Distilled water	200

There is neither clumping nor hæmolysis with this fluid Other solution recommended are Hayem s fluid

Mercuric chloride	0 5 gramm
Sodium chloride	1 0
Sodium sulphate	5 0 grammes
Distilled water	200 c cm

and simple *physiological salt solution* We have not found either of these satisfactory as clumping may occur with the former and hæmolysis with the latter solution

For the *white cell count* we have found the following solution the best —

Glacial acetic acid	2 c cm
Mercuric chloride	0 1 gramme
Aniline gentian violet	one drop
Water	to 100 c cm

The mercuric chloride will prevent growth of moulds when the solution is kept for a long time and the gentian violet will give a slight tinge to the leucocytes and also make it easy to distinguish this solution from the red cell diluting fluid

When the diluting fluids are used a small quantity should be put into a watch glass or other suitable receptacle into which the pipettes charged with blood should be plunged In no case should the pipettes be put into the original phials containing the diluting fluids as there is every possibility that in course of time cells will find their way into this fluid and accumulate at the bottom of the phial these may be taken up subsequently with the diluting fluid and vitiate the count

Enumeration of Erythrocytes

(i) *Filling the pipette* —Before using a pipette see that it is absolutely dry and clean and that the point is intact (the tips are easily damaged) With a dry red cell pipette suck blood up to the mark 0 5 (or 1 0 in the case of anæmic patients) by holding the pipette almost horizontally and at right angles to the line of vision so that the exact height of the column of blood can be seen easily The blood should not go much beyond the mark but if it does go a little beyond it is brought back to the mark by applying the tip of the pipette to the tip of the finger a few times cotton wool or blotting paper should never be used as they will draw out the serum only and the cells in the pipette will become more concentrated The blood adhering to the outside of the pipette is wiped off and the pipette is plunged into the red cell diluting fluid in a watch glass The diluting fluid is drawn up exactly to the mark 101 the pipette being held nearly vertical and gently rotated between the thumb and the forefinger The rubber tube is taken off and a tightly fitting flat rubber band is put round the pipette closing both the ends to prevent any leakage It is now laid flat on the table or in the box

To facilitate counting these 400 small squares are cut up into 16 groups of 16 small squares each by extra lines which are drawn through every fifth square Red cells in the five small groups of squares one from each corner E F G and H and one from the centre J that is to say the contents of 80 small squares in all are counted

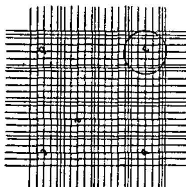


FIG 5—Thoma ruling as seen with two third lens The black circle shows the field seen with the one sixth lens

Counting chamber with Thoma ruling

The disposition of the different part of the Thoma counting chamber is exactly similar to that of the Neubauer counting chamber with this difference however that there is only one big square on each of the ruled areas B B in place of the nine big squares in the Neubauer type The area of each of the big squares is one square millimetre and each is subdivided into 400 small squares each of which is therefore $\frac{1}{400}$ of square millimetre To facilitate counting these 400 small squares are cut up into 16 groups of 16 small squares each by extra lines drawn through every fifth square The square millimetre is thus an exact replica of the central square millimetre in the Neubauer ruling

The coverslip—Only the coverslip which is specially designed for blood-counting chambers must be used If any other coverslip is used there may not be uniform depth in the counting chamber and the count will be inaccurate

The blood counting pipettes
(Thoma type)

The capillary portion of both the red and white cell pipettes (figure 5) is divided into ten equal parts from the tip to the bottom of the bulb the fifth and the tenth marks being denoted by figures 0 5 and 1 respectively There is another mark just above the bulb where the figure 101 is given on the red cell pipette and the figure 11 on the white cell pipette

To facilitate mixing there is a bead in the bulb which is sometimes coloured red in the red cell pipette to distinguish it from the white cell pipette

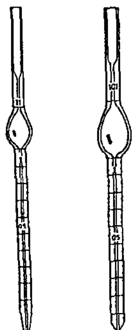


FIG 6—Counting pipettes

objective) may then be substituted by simply rotating the nose piece of the microscope and readjusting the condenser and the diaphragm

Counting the cells

Focus the ruled area with the low power and also find out if the cells are evenly distributed—if not another preparation should be made

The cells in the squares can now be counted with the high power one sixth objective The area from which the cells are to be counted will vary with the different types of ruling but the same rule for cells on boundary lines should hold good in all cases

Rules for cells on boundary lines.—All the corpuscles touching the upper line and the left hand line of a square are considered to be inside the square while those touching the lower line and the right hand line of a square are considered to be outside the square (figure 7)

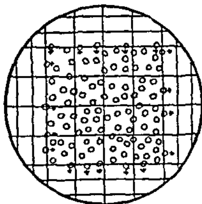


FIG 7 —One field with one sixth lens showing which cells should be counted and which neglected

The cells contained on the five groups of small squares I F G H and J in the middle of the ruled area of the Neubauer ruling (figure 4) are counted

In all cases the cells on both sides of the chamber should be counted and the mean of the two taken Should there be a difference of more than 5 per cent in the two counts the chamber should be recharged and the count done again

Calculations for erythrocyte count

Each small square $=\frac{1}{400}$ square millimetre

Therefore the cubic dimensions of each $=\frac{1}{400}$ $\times 0\ 1$ (space between the cover slip and the ruled area) $=\frac{1}{4000}$ cubic millimetre

And each group of 16 small squares $=\frac{16}{4000}=\frac{1}{250}$ cubic millimetre

Therefore cells from five groups of 16 squares each $=\frac{5}{250}=\frac{1}{50}$ cubic millimetre of diluted blood

Thus the total number of cells counted from five groups of small square multiplied by 50 will give the number of cells in 1 c mm of diluted blood

This when multiplied by the diluting factor 200 (or 100) will give the number of cells in one cubic millimetre of blood

Exampl —Blood 1 taken up to 0 5 mark

Total number of cells counted in five groups of 16 small squares = 450

Therefore 450 × 50 = 22 500 cells per c mm of diluted blood and 22 500 × 200 = 4 500 000 cells per c mm of undiluted blood

When the pipette is filled it will be seen that the entire capillary end of the pipette from the tip to mark 1 is occupied by diluting fluid Hence the true dilution of cells is not 0 5 (or 1) in 101 but 0 5 (or 1) in 100 i e 1 in 200 (or 1 in 100) Thus by taking blood up to the different marks from 0 1 to 1 in the pipette dilutions ranging from 1 in 1 000 to 1 in 100 can be made

Should there be any air bubbles at any stage during the filling of the tube with the blood or the diluting fluid the whole preparation must be rejected and another pipette filled up for it is never possible to get all the air out without loss of some fluid

(*ii*) *Shaking the pipette* —On every occasion prior to charging the counting chamber with the diluted blood the pipette should be shaken preferably in a shaking machine or by rotating the tube held horizontally between the palms of the hands first with the right hand on top and then the left for about 2 minutes The pipette should never be shaken in a horizontal direction i e in the direction of its long axis as this tends to throw the cells into the capillary tube

(*iii*) *Charging the counting chamber with Neubauer ruling* —The counting *chamber and the coverslip must be perfectly clean and free from dust or threads of* cotton Put the coverslip on the counting chamber so that it covers the ruled areas B and B (figure 2) and with the finger nail tap the coverslip to ensure good contact Discard the first few drops of the diluted blood by blowing into the pipette Next holding the pipette in an inclined position of about 45 degrees apply a small drop of diluted blood to that part of the platform A A of the counting chamber which just projects from the coverslip the drop will instantly pass under *the coverslip by capillary attraction The drop of blood should not be too large* or it will overflow the chamber and pass into the overflow trenches D D and may even pass to the transverse trench D Neither should the drop be too small as in that case it will not completely fill the counting surface With a little practice a drop of the requisite volume can be put under the coverslip any little excess on the platform should be removed by sucking with the pipette and never with blotting paper

Small air bubbles are liable to be introduced into the counting chamber together with the diluted blood —

(*i*) if the counting surface and the coverslip are not previously cleaned with the requisite care or

(*ii*) if the point of the pipette is chipped

If there be any overflow into the trenches or if there are any air bubbles remaining the chamber must be cleaned and charged again

Place the counting chamber on the microscope stage which should be on a horizontal plane for about 2 to 3 minutes to allow the cells to settle properly

Focussing the ruled area

Great care is necessary in locating the ruled area without injuring the objective or disturbing the distribution of cells This is most conveniently done by locating the ruled area first with the low power (two thirds objective and 10 x eyepiece)—lowering the condenser and reducing the light by means of the iris diaphragm will show up the rulings distinctly) the higher power (one sixth

TABLE V

Showing the normal red cell counts of different populations

Sex	Age	Locality	Economic status	Number	Mean red cell per C mm in millions	Standard deviation	Authority
Males	19-30	Bombay	Students etc	121	5 110	± 0 380	Sokhey *et al* 1937
	25 45	Calcutta	Mixed	50	5 362	± 0 633	Napier and Das Gupta 1935*a*
	20-45			30	5 533	± 0 490	1936
	19-30	Assam	Coolies	24	5 353	± 0 620	1935*b*
	19 30			0	5 270	± 0 710	1936
		Cachar		25	5 057	± 0 563	Napier and Majumdar 1938
	Adults	U S A			5 400		Castle and Minot 1936
		Britain			5 690		Whitby and Britton 1939
					5 428		Price-Jones 1931
Females non pregnant	16 30	Bombay	Middle class	101	4 470	± 0 330	Sokhey *et al* 1938
	14 38	Calcutta		125	4 615	± 0 409	Napier Edwards and Das Gupta 1941
	17 30	Delhi	Middle class	101	4 560	± 0 250	Benjamin 1939
		Cachar	Coolies	25	4 454	± 0 705	Napier and Majumdar 1938
		Assam		20	4 550	± 0 650	Napier and Bilimoria 1937
		Britain			4 800		Whitby and Britton 1939
					5 012		Price Jones 1931
	18 22	Michigan	Students	50	4 750		Bethel 1936
Females pregnant		Assam	Coolies	40	4 650	± 0 620	Napier and Bilimoria (1937)
		Michigan		28	4 120		Bethel (1936)

When as in the above case blood is taken to the 0 5 mark and the dilution is 1 in 200 the calculation can be simplified by adding four 0 s to the total number of cells counted in the five small squares e g in this instance 450 × 10 000 = 4 500 000

For high counts as in polycythæmia

1 in 200 dilution is not enough as the cells are numerous and would be too crowded to allow an accurate count to be made so the blood is drawn up to the 0 2 mark and the diluting fluid as before up to the 101 mark this produces a dilution of 1 in 500 and the total number of cells in the five squares must be multiplied by 25 000 instead of 10 000 as in the example above

Conversely in an anæmic blood it is advisable to take blood up to the 1 0 mark the dilution will be 1 in 100 and the total number of cells will be multiplied by 5 000

A table (IV) which will facilitate calculation when blood is taken up to the different marks of the red cell pipette and cells from the five groups of small squares of the Neubaüer rulings are counted is given below

TABLE IV

Blood taken	Dilution	Multiplication factor to arrive at number of cells per c mm
0 1 mark	1 1 000	× 50 000
0 2	1 500	× 25 000
0 4	1 250	× 12 500
0 5	1 200	× 10 000
1 0	1 100	× 5 000

With Thoma Ruling Calculation for Erythrocytes

Count the cells contained in the five groups of small squares a b c d and e (fig 5) on both sides of the counting chamber and take the mean of the two count

As the area of each of the small squares is the same as in the Neubauer ruling i e $\frac{1}{400}$ square millimetre the calculations are exactly the same as described for Neubauer ruling

Normal standards

The number of red cells per c mm is usually given in textbooks as 5 000 000 This figure is too low for men and too high for women The mean of a number of counts in different populations is given in table VI

It will be seen that there is a striking uniformity in the counts in different populations compared for example with the hæmoglobin estimations in the same range of populations

practically impo sible In such a case blood is taken up to the 0 1 or 0 2 mark of a white cell pipette (or up to the 0 5 or 1 0 mark of a red cell pipette) and is filled up with white cell diluting fluid The cells are counted from the four big quares as in the ordinary leucocyte count and the number of cells per cubic millimetre is calculated

A table (VI) for facilitating calculation when blood is taken up to the different marks of the pipette and the cells in four areas of 1 square millimetre each are counted is given below —

TABLE VI

Blood taken	Dilution	Multiplication factor to arrive at number of cells per c mm
0 5 in red cell pipette	1 200	× 500
1 0	1 100	× 250
1 white	1 100	× 250
0 2	1 50	× 125
0 4	1 25	× 62 5
0 5	1 20	× 50
1 0	1 10	× 25

WITH THOMA RULING CALCULATIONS FOR LEUCOCYTES

tes in the whole of the cross ruled area are counted at least s should be made and the average count taken

square millimetre represents a cubic capacity of 0 1 cubic hen multiplied by 10 gives the number of cells per cubic ted blood and when again multiplied by the diluting factor e number of cells per cubic millimetre of whole blood

e number of cells multiplied by 100 (when the dilution is 1 10) or 1 the dilution is 1 20) gives the number of cells per cubic millimetre blood

Counting by field method for low counts

This method is suitable in kala azar or other diseases in which there is usually a leucopænia Take blood up to 0 5 mark of the white cell pipette and dilute it 1 in 20

with 5 × eyepiece and one sixth objective focus the square in the centre of the field of the counting chamber with the Thoma ruling or the centre block of the Neubauer and draw out the tube until the diameter of the field measures

Enumeration of leucocytes

For enumeration of the leucocytes follow the technique given under enumeration of erythrocytes taking all the same precautions in the different steps

With a dry white cell pipette suck blood up to the mark 0 5 (or 1 in cases of leucopænia) wipe off the blood adhering to the outside of the pipette plunge it into the white cell diluting fluid and draw the mixture up to the mark 11 take off the tube and put a tightly fitting flat rubber band round to close both the ends, and lay it flat on the table

Here too the capillary end up to the 1 mark of the pipette is occupied entirely by the diluting fluid Hence the true dilution of the cells is 0 5 (or 1) in 10 i e 1 in 20 (or 1 in 10)

By taking blood up to the different marks from 0 1 to 1 in the pipette dilutions ranging from 1 in 100 to 1 in 10 can be made

Counting the cells and calculation to find out the cells per c mm

WITH NEUBAUER RULING

Count the cells in the four corner areas A B C and D (fig 4)

Divide by 4 to get the average number of cells in each area

We know the area is 1 square millimetre and it is 0 1 millimetre deep

Therefore the cubic capacity of each area i 0·1 cubic millimetre (1 × 0 1)

Thus the cells counted are from 0 1 cubic millimetre of diluted blood

This when multiplied by 10 gives the number of cells per cubic millimetre of diluted blood Again this figure multiplied by the diluting factor 20 (or 10) gives the number of cells per cubic millimetre of whole blood

Example —Blood was taken up to 0 5 mark
Number of cells counted from the four squares was 1 0
Therefore the average number of cells in 1 square i e 0 1 cubic millimetre of diluted blood is 30
Therefore 300 (30 × 10) is the number of cells in 1 c mm of diluted blood and 6 000 (300 × 20) is the number of cells per c mm of blood

The number of cells can be calculated rapidly by multiplying the total number of cells from the four square by 50 $\left(\text{i e } \frac{20 \times 10}{4}\right)$ when the dilution is 1 in 20 or 25 $\left(\text{i e } \frac{10 \times 10}{4}\right)$ when the dilution is 1 in 10

Thus in the above example 120 is the number of cells from the four squares and the dilution is 1 in 20

Therefore 120 × 50 = 6 000 is the number of cells per c mm of blood

For high counts as in leukæmia

In cases of leukæmia greater dilution of the blood is necessary for a correct count of th cells as otherwise the cells are so crowded that an accurate count i

practically impossible. In such a case blood is taken up to the 0·1 or 0·2 mark of a white cell pipette (or up to the 0·5 or 1·0 mark of a red cell pipette) and is filled up with white cell diluting fluid. The cells are counted from the four big squares as in the ordinary leucocyte count and the number of cells per cubic millimetre is calculated.

A table (VI) for facilitating calculation when blood is taken up to the different marks of the pipette and the cells in four areas of 1 square millimetre each are counted is given below:—

TABLE VI

Blood taken	Dilution	Multiplication factor to arrive at number of cells per c mm
0·5 in red cell pipette	1 : 200	× 500
1·0	1 : 100	× 250
1 white	1 : 100	250
0·2	1 : 50	× 125
0·4	1 : 25	× 62·5
0·5	1 : 20	50
1·0	1 : 10	× 25

WITH THOMA RULING CALCULATIONS FOR LEUCOCYTES

ocytes in the whole of the cross ruled area are counted at lea t unts should be made and the average count taken

of 1 square millimetre represents a cubic capacity of 0·1 cubic when multiplied by 10 gives the number of cell per cubic iluted blood and when again multiplied by the diluting factor e number of cells per cubic millimetre of whole blood

the number of cells multiplied by 100 (when the dilution is 1 : 10) or hen the dilution is 1 : 20) gives the number of cells per cubic millim tre of d blood

Counting by field method for low counts

This method i suitable in kala azar or other diseases in which there is u ually a leucopænia. Take blood up to 0·5 mark of the white cell pipette and dilute it 1 in 20.

with 5× eyepiece and one sixth objective focus the square in the centre of the field of the counting chamber with the Thoma ruling or the centre block of the Neubauer and draw out the tube until the diameter of the field measures

eight times the length of the side of a small squares The area of the *whole field* is now equal to the area of 50 small squares almost exactly $\left(\pi r^2 = \frac{22}{7} \times 16 = 50\ 2857\right)$

Count the number of cells in 40 such different fields

Calculation —The volume of each square is 1/4 000 cubic millimetre and each field contains 50 squares

Thus if the total number of cells in 40 fields is 70, the number of cells per cubic millimetre of blood $= \frac{4\ 000 \times 20}{50 \times 40} \times 70 = 2\ 800$

The same result may be obtained by multiplying the total number of cells in 40 fields by 40 if the dilution is 1 20 or by 20 if the dilution is 1 in 10

With this method cells of a very large area are counted and the multiplying factor is only 40 or 20 and therefore greater accuracy is ensured

Enumeration of the nucleated cells of the marrow

The enumeration of the nucleated cells of the marrow from the material obtained by sternal biopsy is carried out in the same way as that of the leucocyte in cases of leukæmia

Care of instruments

After use the pipettes coverslip and the hæmocytometer chamber must be cleaned thoroughly

Cleaning the pipettes

1 Thoroughly wash out all the diluted blood with water
2 Remove water with absolute alcohol
3 Remove alcohol with ether
4 Pass air through to dry the pipette so that the bead rolls freely in the bulb

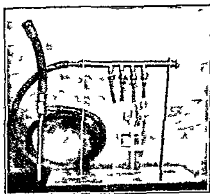


Fig 8 —Suction apparatus for cleaning pipettes

It is very laborious process to clean the pipettes by sucking air through them and one cannot dry them properly by blowing as expired air is laden with moisture The pipettes can be cleaned easily and dried by attaching them to a suction pump (figure 8) or when this is not available by attaching them to a syringe

If the cells clog the tip or any part of the capillary tube loosen them by inserting a stiff horse hair

If there is albuminous matter in the bulb fill it up either with saturated solution of NaOH or potassium bichromate cleaning solution keep overnight in the 37 C incubator and clean next morning

Cleaning counting chamber and coverslip

Wash the ruled side of the counting chamber and the coverslip in running water Thoroughly dry first with a clean cotton handkerchief and finally with a silk handkerchief selvyt cloth or lens paper but avoid rubbing the ruled area of the counting chamber

From time to time the counting chamber and the coverslip should be wiped with alcohol or acetone to remove any grease and then dried with the silk handkerchief but on no account should the counting chamber be immersed or freely cleaned with alcohol

If the rulings become faint after long use the lines may be made prominen by lightly rubbing with a silk cloth on which graphite (lead from a pencil) has been rubbed

TABLE VII

Leucocyte counts in normal individuals

Number	Sex and Age	Residence	Total leucocyte count	Authority
0	Male adult	Calcutta	6 1 ± 1 14	Napier and Das Gupta (unpublished)
1 8	Female adults	Calcutta	16 ± 1 6	Napier Edwards and Das Gupta 1941
198	Male 19—38 years	U S A	,300 (4 400 to 1 400)	Osgood *et al* 193
1	Females 19—36 years	U S A	7 4 0 (4 00 to 13 00)	Osgood *et al* 19

4

ENUMERATION OF RETICULOCYTES

Reticulocytes or reticulated erythrocytes are young red cells they represent the stage just after the cells have lost their nuclei and before they are fully mature The reticulocyte count is expressed as a percentage of the red cells their percentage incidence gives information as to the activity of the red blood cell formation at the moment

The reticulation may appear as a net work over the whole cell as a collection of discrete rods or as granules These are not shown by ordinary Romanowsky stains and a special supravital staining method is necessary demonstrate the reticulation odium blood bout 2

Staining methods.—Various dyes have been tried for staining reticulocytes but brilliant cresyl blue has been found to be the best and it is now used universally. Although brilliant cresyl blue is the dye of choice a number of different methods of using it have been suggested. Methods for reticulocyte staining may be classified in the following groups, *in all of which scrupulously clean and polished slides and coverslips without any scratches should be used** —

A. Methods in which a thin film of the dye is dried on the slide or coverslip and the blood added later.

B. Methods in which the blood is mixed with a solution of the dye.

With either method (*i*) a dried smear or (*ii*) a wet coverslip preparation may be made.

Apparatus required [for method of choice B (ii)]

(*i*) Polished slide
(*ii*) Clean coverslip (preferably [illegible]×40 mm)
(*iii*) Cresyl blue solution 1 per cent in 0.85 per cent sodium chloride
(*iv*) Capillary pipette
(*v*) Rubber teat
(*vi*) Vaseline or paraffin
(*vii*) Gas or spirit lamp
(*viii*) Ehrlich's eye piece (or some means of limiting the microscopic field)
(*ix*) Microscope

Additional requirements for other methods

(*i*) Surgical pricking needle or blood gun
(*ii*) Alcohol, ether and cotton wool
(*iii*) Cresyl blue solution 1 per cent in absolute alcohol
(*iv*) Wide mouthed glass bottle
(*v*) Specimen tube
(*vi*) Filter paper
(*vii*) Leishman's stain
(*viii*) Distilled water

[(iv)–(vi): A (i) and (ii); (vii)–(viii): A (ii) and B (i)]

Method A

To prepare slides or coverslips proceed as follows —

Make a 1 per cent *solution of brilliant cresyl blue in absolute alcohol and* keep it in a well stoppered bottle. The slide may be prepared by one of the following methods —

(a) Take a polished slide, heat it a little over a flame and put it on a flat even surface. With a *capillary pipette take some brilliant cresyl blue*

* *Slides*.—These should be cleaned and polished in the following way —
(*i*) Only new slides should be used. Put the slides in a glass jar containing spirit. Keep them soaked in spirit for over 24 hours.
(*ii*) With a pair of forceps take out one slide at a time, allow the spirit to drain off the slide and then flame it over a spirit lamp or gas burner.
(*iii*) Polish well one side of the slide with jeweller's rouge using a soft cloth. Mark the polished side with a glass pencil and keep it in a dust proof slide box.

Coverslips.—Keep the coverslips in a wide mouthed glass jar soaked in spirit. Just before use with a pair of fine forceps take out one coverslip at a time, allow the spirit to drain off, flame them over a spirit lamp or gas burner, clean with a soft cloth and keep covered in a small Petri dish until they are wanted.

solution from the stoppered phial and put a drop on the middle of the slide If the slide is clean and it is on an even surface the stain will spread concentrically and will be distributed in a uniform manner The stain will dry in a minute or so the slide is then ready for use

(*b*) Put some stain in a wide mouthed glass bottle Warm a polished slide by passing it through a Bunsen flame and holding it in a pair of forceps dip it vertically into the jar of the dye up to three quarter of its length Take it out allow the excess of stain to drain back into the bottle and before it is dry put it in an upright position inside a specimen tube at the bottom of which a piece of blotting paper has already been placed Put in the cork and allow it to stay overnight take it out and wipe the stain from the unpolished side of the slide with a moist cloth it is then ready for use

Slides prepared in either of these ways may be kept for a long time in a dust proof slide box and used when required

(*i*) To prepare a dried smear only prepared by the second methods are suitable

Put a drop of blood oxalated or from the finger on one end of a prepared slide and draw a very thin smear across the stained surface of the slide in the usual way—the use of a hæmocytometer coverslip as a spreader facilities the drawing of a thin smear Slides prepared in this way may be counterstained with any of the Romanowsky stains in the usual way

(*ii*) To make wet coverslip preparations slides prepared in either of the above methods may be used

Put a drop of blood oxalated or from the finger on the stained surface of prepared slide gently place a coverslip on the drop of blood avoiding air bubble as far as possible If the drop of blood is of the proper size it will spread uniformly under the pressure of the coverslip but in case it does not do so apply very gentle pressure with the tip of the finger so that the blood spreads evenly but on no account should the coverslip be pressed hard as some of the cells may be ruptured Ring the coverslip round with hard paraffin by dipping a match in melted paraffin and stroking it on to the slide at the junction of the slide and coverslip

Method B

In this method small amount of blood is mixed with a quantity of dye in some definite proportion

(*i*) Method of Osgood and Wilhelm (1934)

Put 0 5 c cm of 1 per cent brilliant cresyl blue solution in 0 85 per cent sodium chloride into a small test tube on a rack Add an equal quantity of oxalated blood from a well shaken flask Mix well and allow the mixture to stand for about 2 minutes Rotate the tube between the hand to get an even mixture

Withdraw a little of the mixture with a capillary pipette and put a drop at one end of a polished slide Make a thin smear and when it is quite dry counterstain with Leishman s stain in the usual way The slide is now ready for examination for reticulocytes A differential count of the leucocytes can also be done at the same time

(ii) Modification of the above method *This is the method of choice*

Prepare a mixture of cresyl blue and oxalated blood as in the above method With a capillary pipette take out a little mixture and put a small drop at about the centre of the polished side of a clean slide Apply a clean coverslip on the drop of the mixture when under the pressure of the coverslip the mixture will spread evenly but in case it does not do so very gentle pressure with the tip of the finger may be applied over the coverslip The preparation is then sealed with vaseline or paraffin and is ready for examination for reticulocytes only

Counting the reticulocytes

Slides prepared by any of the methods described above are first examined rapidly with a low power (two thirds objective) and a portion of the slide is selected where the cells appear discrete and there are not too many in one field This portion of the slide is now examined with the one twelfth oil immersion lens The total number of red cells in the field and also the number showing reticulation are counted The use of an Ehrlich s eyepiece or a piece of metal with a square hole in it placed inside the eyepiece to narrow down the field greatly facilities the counting Altogether one thousand red cells are counted in a more or less normal case but if the count is high 5 per cent or more 500 red cells are sufficient the number of cells showing reticulation is noted and from this the number of reticulocytes per 100 red cells is calculated

A Veeder counter or some similar device is useful here one counts the red cells and each time a reticulocyte is encountered clicks the Veeder When a thousand red cells have been counted the number on the Veeder is read

In the absence of an automatic counter a pencil and paper must be used for it is a mistake to try to keep two sets of figures in one s head

Comment

Osgood and others have shown that divergent results are obtained with the different methods in use They obtained the best results with the method described by them which has the additional advantage that a differential count of the leucocytes can also be done in the same preparation We have however found that the modified Osgood method which is the routine procedure in our laboratory has given even better results in our hands than the original method With the modified method all the reticulocytes stain very well and there is no clumping nor overlapping of the cells thus the counting is very much facilitated With the original method a number of cells appear broken more so in the case of anæmic blood and the cells

are less discrete The percentage of reticulocytes appears to be slightly lower in the original than in the modified method In a small number of cases where the counts were done by both methods 5 16 per cent reticulocytes were found by the original method against 5 98 by the modified method

On account of the divergent results obtained by the different methods in use for comparative studies a uniform technique should be adhered to in the enumeration of the reticulocytes not only by one group of workers but by all so that the results of different workers will be comparable

The modified Osgood method described above is simple and as it appears to be superior to the other methods in many ways we recommend it as the method of choice for the enumeration of reticulocytes

Normal values

It is unnecessary to quote the figures of numerous observers regarding the normal percentage of reticulocytes When in a healthy individual whose red cells are at the normal physiological level the hæmopoietic and hæmolytic tissues are functioning normally the number of reticulated red cells reaching the peripheral blood is less than 1 per cent of the total red cells The normal range is usually given as 0 1 to 1 0 per cent The present writers found a mean percentage of 0 67 ± 0 37 in a series of 50 city dwelling Indian males with a range from 0 2 to 1 4 and 0 37 ± 0 27 amongst 122 women On the other hand for Assam tea garden coolies the mean was 2 17 ± 1 92 and the range 0 1 to 10 8 per cent It is probably true to say that reticulocyte counts in man of over 1 0 per cent are evidence of some unusual stimulation to the hæmopoietic system though they may be encountered in an apparently healthy individual In our Assam coolie series we accepted these findings of unusually high reticulocyte percentages associated as they were with a low hæmoglobin percentage as evidence of some abnormality of the erythorn in the individuals concerned

Significance of reticulocytes

Whenever the hæmopoietic system meets an extraordinary demand for red blood cells the reticulocyte percentage will rise but the reticulocyte rise will *not* be maintained even though it will take some time for the deficiency of the red cells to be made good

For example if a healthy individual loses a large quantity of blood there is an immediate steady rise in the reticulocyte curve which reaches its maximum and falls to nomal again within a few days whereas it will probably be a few week before the loss of blood cells is made up

Similarly in deficiency anæmias when the deficient substances e g hæmopoietin in pernicious anæmia and iron in iron deficiency anæmia are supplied the hæmopoietic tissues in the bone marrow are now enabled to meet the demand for

red cells and there is a rise in the reticulocyte percentage that starts within two or three days and reaches its maximum within five to ten days after which it falls as fast as it rose

This reticulocyte rise gives a valuable indication that a deficiency has been made good

A third example is in toxic aplastic anæmia where a toxin is depressing the *function of the bone marrow* *In this case there may be no reticulocytes at all to* be found in the peripheral blood but once the toxin is neutralized or otherwise ceases to exert its toxic effect the hæmopoietic tissues will start to function properly reticulocytes will appear in the blood their percentage will rise to a maximum within a few days and will fall again to the normal level but in the absence of any further intoxication the red cells will continue to increase until they reach their nomal level

It should be *emphasised that whilst a reticulocyte crisis as this sharp rise is* called is evidence that the hæmopoietic tissues are functioning effectively a fall of reticulocytes to the normal level does not indicate any cessasion of this functioning but on the contrary it usually indicates that red blood cell formation is being carried on in an orderly manner

In true hæmolytic anæmias there is a constant reticulocytosis as not only will the anoxæmia stimulate erythrogenesis but there is a second source of stimulation in the debris of abnormal hæmolysis which has to be disposed of by the reticuloendothelial tissues

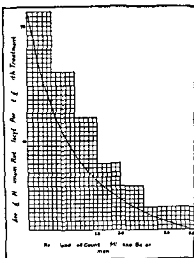


Fig 9—Riddle s chart for calculating the maximum reticulocyte response expected in pernicious anæmia with adequate therapy

A constant reticulocyte count of 5 to 10 per *cent or higher is evidence of excessive* hæmolysis and in a hæmolytic anaemia the return of the reticulocyte count to normal is usually evidence that the excessive blood destruction has ceased

Reticulocyte crises

The extent of the reticulocyte response is governed mainly by the original level of the red cells in the anæmic state For example in a case of pernicious anæmia the rise in the reticulocyte percentage after the same adequate dose of liver extract may be as high as 55 per cent or practically negligible according to whether the red cell count was 500 000 per c mm or 3 000 000 per c mm before treatment Doubling the dose of liver extract would make no difference to the height of the reticulocyte response but reducing it to an inadequate dose would Therefore in pernicious anæmia if *one* has a chart showing the expected reticulocyte response one can

judge whether or not the dose of liver extract given was an adequate dose We have given a chart here not so much for practical use as pernicious anæmia is rare in India but to demonstrate this point

In iron deficiency anæmia the response to adequate iron therapy is also proportionate to the degree of the anæmia but in this case the reticulocyte count does not respond with the same mathematical precision

5
ENUMERATION OF THROMBOCYTES

Thrombocytes or blood platelets are colourless or slightly bluish bodies spherical ovoid or pear shaped and are usually about one third of the diameter of a red blood cell but they may be larger especially in pathological conditions

No method of enumeration of blood platelets is entirely satisfactory as platelets tend to form clumps and to stick to any foreign surface with which they may come into contact However with careful technique counts sufficiently accurate for practical purposes are not difficult to obtain The principal point to be remembered in the platelet count is that manipulation of the sample of blood should be avoided and if possible the blood should be taken directly on to the counting slide

The platelet count is expressed as a number per cubic millimetre of blood There are several methods for estimating the number of platelets these fall under the two main heads the direct and the indirect methods

Apparatus required [for method of choice B (i)]

As in the reticulocyte count

Additional requirements for other methods

For method A (i)—Red blood cell pipette
(ii) Sodium citrate 2 per cent solution (fresh and sterilized)
(iii) Counting chamber with Neubauer ruling and coverslip
For method B (i)—Magnesium sulphate 14 per cent solution

A *Direct method*—In this method the number of platelets per cubic millimetre is calculated without any reference to the red blood cell count

Prick the finger with a sharp surgical needle and with a red cell pipette draw blood up to the 0.5 mark and dilute it with fresh sterile 2 per cent solution of sodium citrate up to the 101 mark After gentle shaking put a drop in the counting chamber wait 3 to 4 minutes to allow the platelets to settle down focus the light carefully and count with the high power all the platelets in 1 square millimetre area The number of platelets per cubic millimetre of blood is then calculated in the usual way

B *Indirect method* —As in the enumeration of the reticulocytes the platelets are at first expressed as a percentage of the red cells calculated from the number of platelets observed in counting one thousand red cells The number of platelets per cubic millimetre can then be calculated from the total red cell count which must be done at the same time Of the many indirect methods advocated the following two methods have given consistently good result in our hands —

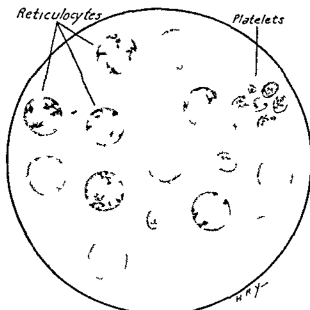


FIG 10—Showing six reticulocytes seven mature erythrocytes and nine platelets

(i) Clean the tip of the finger first with soap and water and then with alcohol and ether and apply a thin layer of sterilized vaseline over it Prick the finger with a blood gun or sharp pricking needle through the thin layer of vaseline As the blood comes out touch the uppermost portion of the blood drop with the centre of a 22 × 40 mm coverslip Place the coverslip on a dried cresyl blue stained slide prepared by method A (*a*) or (*b*) as described for the reticulocyte count With very gentle pressure try to obtain as uniformly spread a film as possible and finally seal the sides of the coverglass with vaseline or paraffin

(ii) Clean the tip of the finger as above Balance a drop of 14 per cent solution of magnesium sulphate on the back of the finger just above and to one side of the nail groove Make a small puncture through the drop The drop of blood should be such that the resultant blood mixture is about 1 in 20 Mix rapidly with a paraffined* capillary pipette Transfer the blood mixture to clean slides make very thin smears and stain with Wright s or Leishman s stain

The coverslip preparation (i) or the slide (ii) is examined with an oil immersion lens A small rectangular field is more convenient for counting than a big circular one (*v* *s*) Note the number of platelets seen in counting 1 000 red cells

Successive fields from the different areas of the smear should be examined so as to eliminate as far as possible the error from uneven distribution

The red cell count is done as a separate procedure in the usual way

* Platelets do not adhere to a paraffined surface so that if glass is covered with a thin layer of paraffin the platelets do not tend to adhere to it The pipette is prepared by drawing hot melted paraffin wax through it a thin layer of paraffin will adhere to the glass when it cools

By a simple calculation the total number of platelets per c mm may now be ascertained Thus if the total red cell count is 5 000 000 per c mm and if x be the number of platelets counted against 1 000 red cells then $\frac{x \times 5\,000\,000}{1\,000}$ or $x \times 5\,000$ is the total number of plateless per c mm

Example —Total red blood cells = 4 500 000 per c mm
Number of platelets counted against 1 000 red cells = 50
Therefore platelets per c mm = $\frac{50 \times 4\,500\,000}{1\,000}$ or $50 \times 4\,500 = 225\,000$

The enumeration of the platelets by the indirect method though more laborious and time absorbing as a total red cell count has also to be made is nevertheless much superior to the direct method Results obtained by indirect methods especially by method (i) are always higher than those of the direct method as there is very little contact with foreign surfaces

Normal values

The figures given by different writers vary considerably This variation is undoubtedly due to some extent to the differences in the methods adopted for counting them but there are considerable variations in th platelet counts done at different times by the same method in the same individual

Whitby and Britton (1939) in Great Britain give the range as 250 000 to 500 000 per cubic millimetre and Gram (1920) in America as 280 000 to 540 000 with the average at 350 000 The present writers found 360 000 ± 248 000 in Calcutta and 423 000 ± 343 000 in Assam in the latter series there were a number of so called normal individuals with platelet counts of over a million and a half

6
DETERMINATION OF CORPUSCULAR VOLUME

Principle —A volume of uncoagulated blood is centrifuged at a high speed in a graduated tube until there is no further packing of the corpuscles The level of packed red cells and of the serum are read off directly from the tube from this the proportion of the packed cells to the whole blood is calculated and is expressed as a percentage

Apparatus required

(i) Graduated cell volume tubes fitted with rubber corks
(ii) Capillary pipettes with rubber teats
(iii) High speed centrifuge machine that will rotate at at least 3 000 revolutions per minute
(iv) A balance to ensure that the weight of the tubes and their contents are about equal
(v) Hand lens

Cell volume tubes—Various types of tube are in use these vary in length bore and capacity Wintrobe uses small tubes 11 centimetres long with a bore of 2 5 millimetres which are marked in millimetres and centimetres from 0 to 10 In our laboratory we use a tube 7 5 centimetres long with a bore of 7 millimetres and graduated from 0 to 110 These tubes were specially made for us by Messrs Baird and Tatlock London to fit in our high speed centrifuge machine but comparatively short graduated tubes of this kind are now easily obtainable from instrument makers

As the degree of packing of the cells depends to a certain extent on the calibre of the tube it is desirable that the same set of tubes which were used in working out the normal standards should be used in all subsequent work

Method—The blood is taken in the usual way into a 5 c cm or 3 c cm flask containing oxalate (*see* p 2) With a capillary pipette withdraw oxalated blood from the flask after it has been well shaken fill up the graduated cell volume tube from the bottom upwards exactly up to the 100 mark put in a small rubber cork to prevent any evaporation during centrifugalizaion and place it in the centrifuge bucket

If possible two specimens should be placed in the centrifuge at the same time In any case the centrifuge bucket containing the graduated tube must be balanced against a similar bucket and tube so that their weights are exactly equal before these two buckets are placed in opposite sides of the centrifuge this elementary principle must be observed rigidly both for the sake of the centrifuge and for satisfactory packing Centrifuge at a high speed—2 500 to 3 000 revolutions per minute—until there is no further packing of the red cells

The maximum time required for complete packing of the red cells must be found out by a few preliminary experiments This will depend to a great extent on the centrifuge machine

The tubes are first spun for 20 minutes at a high speed the actual speed being noted they are taken out and the readings taken they are again spun for another 5 minutes and the readings again taken The process is continued until there is no difference between two consecutive readings—the time at which the first of these two readings was taken is the time required for complete packing of the cells as there was no further packing The mean time in minutes for 10 to 20 such experiments is taken as the time required for complete packing of cells with that particular centrifuge machine at that particular speed However in all subsequent examinations a little extra time say 5 minutes should be allowed over and above the mean time determined

Generally the cells are fully packed if the tubes are spun for about 20 minutes in a centrifuge machine at a speed of 2 500 to 3 000 revolutions per minute but it is safer to allow 30 minutes

The tubes are now taken out of the buckets and with a hand lens the marking on the tube corresponding to the level of the packed red cells is noted

also the top level of the serum is checked If this is at the mark 100 then the red cell reading can be translated directly into a percentage but if it is more or less than 100 the percentage figure must be calculated making an allowance for this fact

Example—If the top of the serum is at the 102 level and the red cells at 42 the cell volume is $\frac{100}{102} \times 42 = 41.17$ per cent

With leukæmic blood it is almost impossible to read the top level of the red cell accurately and therefore it is difficult to ascertain the correct cell volume This may be remedied at least partially by allowing the charged cell volume to stand for half an hour before centrifuging when the demarcation between the red and white cells levels will be more clearly seen

Anti coagulant.—This has been discussed above (p 2) but perhaps we should again mention that if the isotonic mixture of potassium and ammonium which we recommended be used no shrinkage will occur

On the other hand if potassium oxalate alone (0 2 per cent) be used then a factor × 1 09 must be applied to the cell volume before the corpuscular value are calculated

Example—The uncorrected cell volume is 44 per cent the corrected cell volume will be $44 \times 1.09 = 47.96$

CALCULATION OF CORPUSCULAR VALUES

From the red cell count the hæmoglobin expressed in grammes per cent and the corpuscular volume certain absoulte values can be calculated

These values are —

(1) the mean corpuscular volume (MCV)

(2) the mean corpuscular hæmoglobin (MCH) and

(3) the mean corpuscular hæmoglobin concentration (MCHC)

Mean corpuscular volume

This is the mean or average volume of a single red cell

It is expressed in cubic microns (cu μ) and is obtained by the following method —

$$MCV = \frac{\text{Volume of packed red cells in c cm per 1 000 c cm of blood}}{\text{Red cells in millions per c mm}}$$

Example—Corpuscular volume = 40 c cm per 100 c cm of blood
Red cells count = 5 000 000 per c mm

$$\text{Mean corpuscular volume} = \frac{40 \times 10}{5} = 80.0 \text{ cubic microns (cu } \mu)$$

Mean corpuscular hæmoglobin

This is the average hæmoglobin content of a single red cell expressed in micro micrograms ($\gamma\gamma$)

It is obtained by the following method —

$$MCH = \frac{\text{Hæmoglobin in grammes per 1 000 c cm of blood}}{\text{Red cells in millions per c mm}}$$

Example —Hæmoglobin = 14 grammes per 100 c cm of blood
Red cells = 5 000 000 per c mm

$$\text{Mean corpuscular hæmoglobin} = \frac{14 \times 10}{5} = 28\ 0\gamma\gamma$$

Mean corpuscular hæmoglobin concentration

This is the mean or average of the hæmoglobin concentration in each cell and is expressed as a percentage of the cell contents —

$$MCHC = \frac{\text{Hæmoglobin in grammes per 100 c cm of blood}}{\text{Volume of packed cells in c cm per 100 c cm of of blood}} \times 100$$

Example —Hæmoglobin = 14 grammes per 100 c mm of blood
Corpuscular volume = 40 c cm

$$\text{Mean corpuscular hæmoglobin concentration} = \frac{14}{40} \times 100 = 35\ 0 \text{ per cent}$$

That is to say in this instance 35 per cent of the cell substance consists of hæmoglobin

The great advantage of these mean values is that in calculating them it is not *necessary to decide on any arbitrary normal as one does in calculating indices e g* the colour index for arriving at which one usually considers 5 000 000 red cells per c mm and 100 per cent hæmoglobin to be the normal values Unqualified a colour index is a meaningless expression as even if the above normal values have been taken it is still uncertain what 100 per cent hæmoglobin means (*vide* p 5) If *later* one decides on the normal values for the particular population then it is a very simple matter to calculate the colour index

Example —MCH of the particular case = $35\gamma\gamma$
Mean MCH of the population = $30\gamma\gamma$

$$\text{Colour index (CI)} = \frac{35}{30} = 1\ 16 = 1\ 17$$

Similarly the volume index and the saturation index can be calculated from the MCV and MCHC respectively

There is in our opinion little point in calculating these indices and we recommend workers to get into the habit of thinking in terms of *mean corpuscular values* instead of indices just as they should get into the way of thinking in terms of grammes of hæmoglobin instead of percentages

Normal values —There are undoubtedly different normal values for different populations We will consider mean corpuscular volume first Whitby and Britton give 86 as the mean with the range from 80 to 94 but neither these writers nor the majority of others who give a normal range make any attempt to explain what they mean by the normal range They can scarcely mean that *all* normal observations fall within the range but they do not specify what percentage of observations may

5

TABLE VIII
Corpuscular values

Sex	Locality	Number	Mean corpuscular volume	Standard deviation	Mean corpuscular hæmoglobin	Standard deviation	Mean corpuscular hæmoglobin concentration	Standard deviation	Authority
Male	Calcutta	30	90 49	± 7 90	28 53	± 2 31	31 07	± 1 0	Napier and Das Gupta 1936
	Bombay	121	87 08		30 01		34 54		Sokhey *et al* 1937
	Assam	74	71 29	± 7 04	23 93	± 2 31	32 50	± 3 10	Napier and Das Gupta 19 6
	Cachar	25	84 93	± 10 78	25 14	± 3 70	29 72	± 2 94	Napier and Majumdar 1938
Unspecified	U S A		87 00		27 5		35 00		Castle and Minot 1936
	Britain		86 00		29 5		34 00		Whitby and Britton 1939
Females	Calcutta	128	86 82	± 7 28	27 42	± 2 89	31 57	± 1 76	Napier Edwards and Das Gupta 1941
	Bombay	101	88 53		9 06		32 86		Sokhey *et al* 1938
	Delhi	100	92 7†		28 ,6		33 58		Benjamin 1939
	Assam	0	77 30	± 7 ,0	4 50	± 3 00	31 20	± 1 70	Napier and Bilimoria 1937
	Cachar	25	82 49	± 12 68	23 42	± 3 10	8 67	± 3 37	Napier and Majumdar 1938
	Michigan	50	86 30						Bethel 1936
Pregnant females	Calcutta	64	86 83	± 10 08	6 62	± 3 34	30 57	± 2 13	Napier and Edwards 1941
	Assam	40	72 10		23 80		32 60		Napier and Bilimoria 1937
	Michigan	28	92 00						Bethel 1936

Calculated from data given after applying factor × 1 09 to cell volume to allow for shrinkage where this has not been done
† Corrected figures recently supplied by worker

be expected to fall within it If one gives the mean (m) and the standard deviation (sd) of a number of observations that at least does mean something definite namely that two thirds of the observations will fall between m + sd and m − sd and that 19 out of 20 will fall between $m + 2 \times sd$ and $m - 2 \times sd$

The last figures seem reasonable ones for practical purposes and can certainly be applied amongst hæmopoietically more stable populations than we usually encounter in India but in most of our populations it provides too wide a range Even for our Calcutta series in which the mean is 90 49 cu μ the range would be (90 49 + 2 × 7 90 =) 106 29 to (90 49 − 2 × 7 90) 74 69 or in round figures from 75 to 106 this is too wide to be considered a normal range though the mean is close to that given by the workers On the other hand some of the Assam so-called normal populations have a very low MCV and if the same rule were applied the range would be ridiculously low e g 57 21 to 85 37 cu μ in the first Assam series quoted but there is evidence in this case that the whole population is suffering from a degree of iron deficiency anæmia therefore as the figures are not based on a truly normal population this rule cannot be applied

It is obvious that no hard and fast rule can be laid down Whenever possible it is best to examine normal individuals of the population concerned and where one can be certain of excluding most of the cases of sub clinical blood dyscrasia to calculate the range from the mean *plus* or *minus* twice the standard deviation Otherwise 80 to 100 cu μ may be taken as the normal range for general use in India That is to say any figure below 80 cu μ may be taken as indicating microcytosis and any above 100 cu μ as indicating macrocytosis

Similarly for the mean corpuscular hæmoglobin on the Calcutta figures the normal range should be 28 53 ± 2 × 2 31 = 23 91 and 33 14 or roughly 24 to 33$\gamma\gamma$ This is in our opinion a good normal range for general use in India any figure below 24$\gamma\gamma$ indicates hypochromia and any above 33$\gamma\gamma$ hyperchromia

Finally a good range for the mean corpuscular hæmoglobin concentration is 30 to 35 *this is not based on any of the data quoted for reasons into which we* need not go now but on our general experience

7

HYPERBILIRUBINÆMIA AND VAN DEN BERGH S TEST

Bilirubin the chief pigment of the bile is a by product of hæmolysis It is derived from hæmoglobin being the porphyrin (globin and iron free) fraction of the hæmoglobin molecule

The bilirubin is formed by the disintegration of the red blood cells by specialised reticulo endothelial cells present in spleen liver bone marrow and elsewhere The result of the disintegration of the red cells is the formation of a simple iron compound which is preserved and re utilised for the formation of new red cells and an iron free pigment bilirubin which is excreted by the liver in the bile It was formerly believed that this disintegration of the red cells and elaboration of bilirubin used to take place in the liver alone but recent researches have definitely

shown that bilirubin is elaborated in any organ having these specialised reticuloendothelial cells but is mostly seen in spleen bone marrow and liver

But wherever it is formed the bilirubin enters the liver through the hepatic vascular capillaries from where it passes through the polygonal cells into the bile capillaries lying in between the column of liver cells and is finally discharged into the bile ducts

Normally, the bilirubin is concentrated in the gall bladder and would pass into the small intestine In the large intestine the bilirubin undergoes reduction by bacteria to form urobilinogen (also called stercobilinogen) A part of the urobilinogen is excreted in the stools and on exposure is converted into urobilin (stercobilin) by oxidation But the bulk of the urobilinogen is re absorbed in the portal circulation passes to the liver and is almost completely re excreted in the bile Any urobilinogen which may escape into the general circulation is excreted by the kidney and is converted to urobilin by oxidation when the urine is voided

As a result of normal hæmolysis of the red blood cells bilirubin is normally present in the plasma and serum usually in a dilution of 1 in 400 000 or 0 25 mgm per 100 c c of blood but may be present up to a dilution of 1 in 100 000 or 1 0 mgm per 100 c c of blood The yellow colour of the plasma and serum is in part due to the presence of bilirubin When bilirubin is present in the blood in excess of the normal amount the condition is known as hyperbilirubinæmia

Ehrlich observed that when sulphanilic acid and sodium nitrite were added to a solution of bilirubin a reddish violet substance azo bilirubin was formed The reaction is specific and very delicate will show bilirubin in a dilution of 1 in 1 500 000

Van den Bergh made use of these observations not only to test for the excess of bilirubin but to draw certain conclusions from the nature of the result His original claims are now questioned but his general conclusions hold good and form the basis of the test which we are describing

The van den Bergh reaction

The reaction is classified into two main types the *direct* and the *indirect* The direct reaction may occur either as *immediate direct* reaction or *delayed direct* reaction A third type of reaction the *biphasic reaction* may also occur

If the passage of the bile is obstructed due to obstruction in the hepatic or common bile duct the secretion of bile continues until the pressure in the ducts equalises the secretory pressure of the liver cells When this occurs the bilirubin would enter the liver but instead of being secreted into the bile capillaries is re absorbed by the liver cells when it combines in some loose way with bile salts and cholestrin and passes out of the liver through the hepatic vein and the lymphatics into the general circulation The association of the bilirubin with bile-salt and cholestrin prevents its adsorption by the proteins of the blood and therefore bilirubin in this state will give a *prompt direct* van den Bergh reaction

When the destruction of the red cells is such that the balance between the production and excretion of the bile pigment cannot be maintained even by a normal liver with its large functional reserve the concentration of the bilirubin

rises above the normal level in the blood. The excess bilirubin does not pass through the liver cells and is in some loose combination with the proteins of the blood and therefore would give a positive reaction with the diazo reagent only when the proteins are precipitated with alcohol. Thus *indirect* van den Bergh test is found to be positive in cases of excessive formation of bilirubin due to increased destruction of a large number of red blood cells.

In conditions of liver dysfunction associated with excessive hæmolysis the *bi phasic* reaction will be positive indicating that both forms of bilirubin are present.

The technical details of the test have been modified many times and it is done in a number of ways in different laboratories. Godfried (1935) recommends the method described by Thannhauser and Anderson (1921) as the most suitable for clinical purposes; this is the method that we follow in our laboratory.

Apparatus required

(i) Graduated pipettes 1 c cm 2 c cm 5 c cm and 2 c cm
(ii) Small test tubes 4 inches by ½ inch
(iii) Centrifuge tubes
(iv) Small glass beakers or flasks
(v) Capillary pipettes with rubber teat
(vi) Centrifuge machine
(vii) Sealed cobalt sulphate standard tubes
(viii) * A Lovibond comparator with the coloured discs for bilirubin

Reagents

Alcohol—absolute and 90 per cent.
Saturated solution of ammonium sulphate; this is prepared by dissolving an excess of ammonium sulphate in hot water (so that some undissolved salt is left at the bottom).
Normal saline
Diazo reagent
(A) Sulphanilic acid—1 gramme
Concentrated hydrochloric acid—15 c cm
Distilled water 1 000 c cm
(B) Sodium nitrite—0 5 gm
Distilled water 100 c cm

Stock solutions of (A) and (B) are made. Separately they keep well for a long time.

The test can be done either with the serum or plasma. When the cell volume is also being estimated, the supernatant plasma in the cell volume tubes, after the packing of the red cells, is quite sufficient for the test. When the test is done by itself, 3 cubic centimetres of blood is drawn and placed in a dry test tube or an oxalated test tube.

The blood is collected in the usual way and even the slightest hæmolysis must be avoided (*vide supra*). Preferably the blood should be collected in the fasting state as it is claimed that food intake, especially a big meal of carbohydrate and fat, affects the level of serum bilirubin. The specimen must always be examined within 2 hours, as otherwise paradoxical results may be obtained.

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* The comparator can be obtained from British Drug House, London, but stocks may be available at this Company's depots in India: Imperial Chemical House, Ballard Estate, Bombay, or c/o H S Clark, Kent House, 33 Mission Row, Calcutta.

Technique —Prepare the diazo reagent by mixing 10 c cm of (A) and 0 3 c cm of (B) in a small flask or beaker

This mixture must be prepared just before use and must never be used later than one hour after it is made

Pipette off the clear supernatant plasma from the cell volume tubes

Take exactly 1 c cm of plasma in a centrifuge tube

Add exactly 0 5 c cm of diazo reagent and mix well

One of the following things may happen —

(*i*) The mixture becomes reddish violet within 30 seconds—*Direct reaction*

(*ii*) The mixture becomes slightly reddish at once and the colour gradually increases in intensity and becomes reddish violet—*Biphasic reaction*

(*iii*) A reddish colour appears in about a minute which gradually deepens into reddish violet —*Delayed direct reaction*

No change of colour occurs within 10 minutes

Indirect reaction —If no change occurs add to the mixture of plasma and diazo reagent 2 5 c cm of alcohol (absolute or 96 per cent) and 1 0 c cm of saturated solution of ammonium sulphate

Mix well by inverting the tube allow it to stand for about 2 minutes and centrifuge for 5 to 10 minutes

A positive *indirect* reaction is indicated by the upernatant fluid becoming coloured a distinct bluish violet the intensity depending on the amount of bilirubin present As bilirubin is present in normal blood a faint violet colour nearly always appears such a reaction obviously has no pathological significance

Quantitative indirect reaction —It will be clear that some form of quantitative estimation is essential Many elaborate methods which require special apparatus have been devised but a fairly accurate estimation of the bilirubin content can be obtained by carrying out the test in the way described above and comparing the supernatant fluid with a prepared standard or with a permanent colour standard

(*i*) Comparing the supernatant coloured fluid with the cobalt sulphate standard (2 161 per cent) has given the most satisfactory colour matching in our hands

Preparation of the cobalt standard solution —Dissolve 2 161 grammes of anhydrous cobaltous sulphate in 100 c cm distilled water The colour of this solution corresponds to that of 1 in 200 000 bilirubin or 0 5 mg of bilirubin per 100 c cm Solutions equivalent to 0 4 0 3 0 2 0 1 and 0 05 mg per 100 c cm are made by diluting the original solution

About 2 c cm of each of these solutions is put into tubes made of hard glass The tubes are then sealed numbered and kept in the dark and are brought out only when required Properly stored the solutions retain their colour for a fairly long time In our laboratory we did not find any difference in colour in solutions which were in daily use for over six month when these were compared with freshly prepared solutions

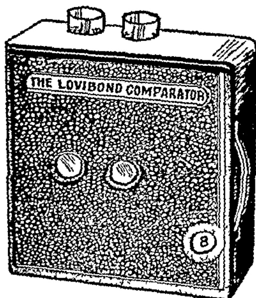
Technique for the use of cobalt tandard solutions —Take about 2 c cm of the coloured supernatant fluid in a tube having identically the same bore as the standard

tubes and compare its colour in a comparator with the cobalt standard tubes The figure on the tube giving the correct matching with the supernatant fluid multiplied by four gives the bilirubin value of the undiluted serum for the dilution of the serum in the supernatant fluid containing azo bilirubin is 1 in 4 and not 1 in 5 because the saturated ammonium sulphate remains as a separate layer and does not contain azo bilirubin

If the supernatant fluid is of a deeper colour than the cobalt standard tube marked 0 5 dilute the supernatant solution with normal saline (dilution with 67 per cent alcohol which is recommended makes the solution hazy and difficult to match) until it matches one of the cobalt standard tubes Note the dilution required *to match and calculate the amount of bilirubin in the specimen*

Example —One cubic centimetre of supernatant fluid was taken to which was added *1 cubic centimetre of normal saline to match the tube marked 0 4*
First dilution of serum in the supernatant fluid is 1 in 4
Second dilution to match the colour is 1 in 2
Therefore total dilution is $\frac{1}{4} \times \frac{1}{2} = \frac{1}{8}$
Then the original plasma contained $0\,4 \times 8 = 3\,2$ mg of bilirubin per 100 c cm

(ii) *Lovibond comparator* —Quantitative estimation of the indirect van den Bergh reaction can be simplified by the use of a bilirubin disc in the Lovibond comparator *The disc contains coloured glass standards marked* 0 2 0 4 0 6 0 8 1 0 1 25 1 50 1 75 and 2 0 mg which are calibrated in such a way that when the test is done in the way described above the matching with any of the discs gives directly the result for the undiluted serum (figure 11)



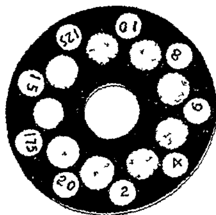


Fig 11

Lovibond comparator with the bilirubin disc and tubes in position

Bilirubin disc for the *Lovibond* comparator

Technique for the use of the comparator.—Fit in the disc marked bilirubin in the Lovibond comparator. Transfer the coloured supernatant fluid to the right hand tube of the comparator. A tube of distilled water should be put behind the hole showing the coloured glass. Hold the comparator against a source of light (daylight is much better than any artificial light). Rotate the disc till a correct colour match is obtained; the number that is visible indicates the bilirubin value in mg. per 100 c.cm. of undiluted serum of plasma, e.g. 0.8 mg. in the above figure.

When the colour of the supernatant fluid appears to lie between two consecutive coloured discs and does not exactly match either of them an intermediate value is recorded.

If the bilirubin value exceeds 2 mg. per 100 c.cm., dilute the supernatant fluid with normal saline until a correct match is obtained with one of the coloured glasses. Calculate the bilirubin value by multiplying the number on the disc by the dilution factor.

Discussion.—The significance of the positive indirect van den Bergh test in hæmatological investigation is that it gives a *broad* indication as to whether the anæmia is due to excessive blood destruction or not.

In the true hæmolytic anæmias the test always gives a high reading, 2.0 milligrammes or more.

In pernicious anæmia a reading well above the normal range is usual, for though it is not a true hæmolytic anæmia, the deficiency of hæmopoietin leads to the formation of abnormal cells which are very susceptible to destruction by the normal hæmolytic tissues.

In tropical macrocytic anæmias in a large number of cases, especially in those with enlarged spleens, the test is much above the normal range and in a small number of cases the test is well within the normal range. This is probably due to destruction of an increased number of red blood cells on account of the proliferation of the reticula endothelial cells in the spleen. The associated liver dysfunction is also a probable factor both in pernicious anæmia and tropical macrolytic anæmia in producing an increased indirect van den Bergh reaction.

In iron deficiency anæmias it is within normal limits, and in aplastic anæmias it may be completely negative.

It is not, however, an absolute indication either of excessive blood destruction or of deficient blood formation, as, for example, in an acute malarial attack, when there may be enough red cells destroyed to lower the red cell count a million or more cells per c.mm., the van den Bergh often remains within normal limits, for the liver cells are able to excrete this temporary excess of bilirubin in the blood, but if the red cell destruction is continued the bilirubinæmia will inevitably rise above the normal range and a positive indirect van den Bergh reaction will result.

On the other hand, in liver dysfunction, not necessarily accompanied by anæmia, a positive van den Bergh may be found, for the liver cells are unable to utilize the products of normal hæmolysis.

TABLE IX

Showing the normal bilirubin according to different observers

Author	Bilirubin in milligrammes per 100 c cm of plasma or serum		
	Range	Mean	S D
van den Bergh (Vaughan and Haslewood 1938)	0 08-0 24		
Greene *et al* (19 5)	0 3 2 0		
Perkin (1927)	0 05-0 35		
Barron (1931)	0 1 0 24		
Elton (Vaughan and Haslewood 1938)	0 0 0 25		
Vaughan and Haslewood (1938)	0 2 1 7	0 54	± 0 5
Mills and Mawson (1938)	0 1 1 0	0 31	± 0 16
Napier and Das Gupta (1941)	0 0 1 0	0 20	± 0 3

8
ICTERUS INDEX

Like the van den Bergh reaction the icterus index is a measure of the bilirubin content of the blood Though the test is simple to carry out it does not give all the information which is obtained by the van den Bergh test

Principle of the test —The colour of the serum or plasma is compared with a standard solution of 1 in 10 000 potassium bichromate which is taken as the unit

Apparatus required

(i) Test tubes in a rack
(ii) Capillary pipettes with rubber teats
(iii) Potassium bichromate solution 1 in 10 000 (to be stored in the dark)
(iv) Colorimeter of the Duboscq or Klett type

Technique —Pipette off the supernatant plasma from the cell volume tube after complete packing of the cells

Dilute the plasma with an equal part or with twice the amount of normal saline (more in cases of jaundice) dilution diminishes the cloudiness of the plasma and gives a better matching

Matching in the colorimeter.—The following adjustments of the scales and of the illumination must always be made each time before the colorimeter is used

(i) Adustment of the scales Gently raise the cups until the bottoms are in contact with the plungers The readings should be exactly at O on both sides If they are not they must be brought to O by adjustment

(ii) Adju tment for uniform illumination Half fill both the cups with the standard 1 10 000 potassium bichromate solution Gently raise the cups until the bottoms touch the plungers—this will drive out any air bubbles that may be in the fluid

Now slowly bring down the cups and set them at an equal depth say 15 millimetres Adjust the reflector in such a way that the same amount of light i reflected up through each cup

Slight adjustment of the eyepiece may be necessary if both halves of the field do not match exactly

Taking a reading.—Keep the cup on the left side at a depth of 15 millimetres Take out the cup from the right side throw out the potassium bichromate solution wash the cup and plunger in water dry well with soft linen Put the diluted plasma into the cup and replace it on the right side of the colorimeter Gently rai e the cup until its bottom touches the plunger Now move this cup containing the plasma up and down until a perfect match is obtained take a reading Move the cup up or down and again adjust it to match take another reading

Take the mean of three independent readings

Calculation

$$\text{Icterus index} = \frac{\text{Reading of standard bichromate solution}}{\text{Reading of diluted plasma solution}} \times \text{dilution factor}$$

Example.—One part of plasma is diluted with two parts of normal saline
The cup containing the standard solut on is at 15 mm
The cup containing the unknown plasma matches the standard at 5 mm

$$\text{Icterus index} = \frac{15}{5} \times 3 = 9$$

B *Matching in tubes*.—In the absence of a colorimeter results sufficiently satisfactory for clinical purposes may be obtained by matching the plasma or serum diluted with normal saline against the standard 1 10 000 potassium bichromate solution

Take two test tubes of equal height and calibre Into one put about 5 to 10 c cm of standard potassium bichromate solution Into the other put 1 c cm of plasma and add measured amounts of normal saline from a graduated pipette until the colour matches the standard

Calculation.—The dilution of the plasma required to match the standard potassium bichromate solution gives the icterus index

Example.—To 1 c cm of plasma was added 7 c cm of normal saline to match the standard
Dilution of the plasma is 1 in 8
Ther fore icterus index = 8

C *Matching in a Lovibond comparator*—If this comparator is available it is an inexpensive matter to obtain an icterus index disc The undiluted serum or plasma is placed in the right hand tube and matched directly against the colour standards in the Lovibond comparator When a correct matching is obtained the icterus index is read from the right hand corner of a comparator as in the case of the van den Bergh test (*vide* figure 11)

The normal icterus index is 4 to 7

9
THE MAKING AND STAINING OF BLOOD FILMS

Properly made and well stained blood smears are essential for the white cell differential count the enumeration of the nuclear lobes of neutrophil polymorpho nuclears as in the Arneth and Schilling counts and also for the determination of the red cell size by the Price Jones method

For ordinary work blood films are made on 3 by 1 inch glass slides and for special studies on 2 cm square coverslips only new slides and coverslips should be used if this is possible The slide and the coverslip must be thin completely transparent scrupulously clean and free from grease and dust

Cleaning glass slides and coverslips

Place the slides and coverslips in sulphuric acid bichromate mixture* and allow them to remain for 24 hours this mixture should be kept in a large jar for the slides and in a small one for the coverslips Decant off the cleaning mixture into another similar glass jar which is then ready for use again Transfer the slides or cover slips to a shallow enamelled tray or a big petri dish and place this under a tap for 3 to 4 hours stirring from time to time with a glass rod Finally wash with distilled water dry with a piece of soft linen and put them in a jar of absolute alcohol for 24 hours (methylated spirit may be used as a substitute) With a pair of forceps take out the slides or coverslips one by one allow the excess of spirit to drain off flame in a bunsen or spirit lamp clean again with soft linen and store in a dust proof container—in a slide box for the slides and in a small petri dish for the coverslips These are now ready for use for all ordinary work

Polishing the slides

For special work polish one side of a slide cleaned as above with jewellers rouge for 2 to 3 minutes clean with soft linen (velvet cloth or silk is the best for this purpose) mark the polished side with a glass pencil and store for future use in a dust proof container

(A) SPREADING THE FILM IN THE ROUTINE USE OF VENOUS BLOOD

(i) *On slides*—Put a few polished slides on a flat surface With a syringe collect the blood from a vein (*vide* p 2) Before putting the blood into the

* Concentrated sulphuric acid	100 c cm
Potassium bichromate (powdered)	100 gm
Water	to 1 litre

oxalated flask and while the needle is still attached to the syringe put a small drop of blood on to the middle of the slide a little away from the end Apply the thin edge of another slide with the corners cut off so that the spreading edge is narrower than the slide or better still of a spreader (hæmocytometer cover slips make excellent spreaders on account of their smooth narrow edge) to the middle of the glass slide slide it along until it makes contact with the drop of blood which will now spread along the edge of the spreader (slight lateral movement will accelerate this) then with the spreader at an angle about 30 to 40 to the slide push the spreader with the blood following it along the slide and raise the spreader abruptly just before the whole drop of blood has been used up The film should be dried quickly by waving it in the air or under an electric fan An ideal film should occupy the middle third of the slide should be of uniform thickness should not have any tails and when viewed under the microscope the red cells should just touch one another and there should not be any rouleaux formation anywhere on the slide



(a) Fig 12 (b)

Figure 12*a* shows a satisfactory film but with tails and figure 12*b* a good film with a straight leucocytic edge They were drawn from above downwards Figure 13 shows the low power view of a satisfactory leucocytic edge the leucocytes are actually discrete though the photograph scarcely gives that impression

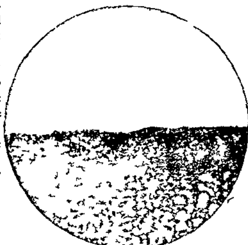


Fig 13

The important points are a perfectly clean slide a good spreader and a drop of blood of the right size this last can only be gauged by trial and error but compared with the size of the drop required from a normal individual a larger drop will be required from an anæmic patient and a smaller drop from one with polycythæmia or leukæmia

(*ii*) *On coverslips* —Place a few clean coverslips on a flat even surface Proceeding as in (*i*) put a very small drop of blood on the centre of a coverslip place another clean coverslip of the same size on the top of the drop of blood in such a way that the sides of the two coverslips are not opposed (*see* figure 14) If the two coverslips are clean the blood will spread uniformly between them Now draw apart the coverslips quickly but gently and dry them quickly by waving them in the air or under an electric fan

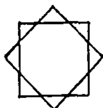


FIG 14

(B) FROM CAPILLARY BLOOD FROM THE FINGER OR EAR LOBE

Prick the finger or ear lobe fairly deeply with a sharp surgical needle or with a blood gun so that the blood flows freely from the wound Apply the surface of a clean slide or coverslip to a small drop of blood and proceed as in A (*i*) or (*ii*)

Comparing the two methods

The method of choice is in our opinion the slide smear The advocates of the coverslip method claim that it is the only way to obtain an even distribution of cells and that consequently reliable differential counts can only be made from coverslips On the other hand it is much easier to make a good film on a slide and if the whole drop of blood on the slide is utilized in making the smear and this drop is a small one examination of such a smear will give as reliable information as examining a coverslip smear the preparation of which demands considerable dexterity

Staining blood films

All blood smears should be stained within 24 hours If the smears cannot be stained immediately they must be fixed with methyl alcohol and stored in a dust proof slide box for staining at a later date The unstained slides must never be left uncovered on the working table as blood is readily eaten by flies during the day and by cockroaches at night

Romanowsky stains are used for all blood work The most commonly used stains are those originated by Leishman Wright Jenner and Giemsa All these stains depend for their action on the compounds formed by the interaction of methylene blue and eosin and the differences between the various stains are due to the proportion of the two stains Excepting Giemsa s stain the fluid stains are prepared by dissolving the dry powder in acetone free pure methyl alcohol so that a preliminary fixation with methyl alcohol is only required in the case of Giemsa s stain Leishman s and Wright s stains are used in the strength of 0 15 per cent and Jenner s stain in the strength of 0 5 per cent

Preparing Leishman s Wright s and Jenner s stains

Stains in powder or tablet forms and extra pure acetone free methyl alcohol for dissolving the stains should be obtained from some reliable firm We have found Gurr s* stains to be very satisfactory

All the glassware used in preparing the stains and in storing them should be scrupulously clean and free from any trace of water they should be rinsed first with absolute alcohol and finally with a little methyl alcohol

Take the requisite amount of stain in powder or tablet form in a mall glass mortar Measure out the requisite amount of methyl alcohol in a graduated glas cylinder Pour out about 2 c cm of methyl alcohol on the stain and grind well to make it into a thin paste Add in small quantities at a time about half the total amount of methyl alcohol grinding all the time Carefully decant the supernatant dissolved stain into a clean glass stoppered bottle Add more methyl alcohol to the undissolved stain grinding as before Again decant the supernatant stain into the bottle continue the process until all the methyl alcohol is used up If this is properly done all the stain will go into solution and no residue will be left at the end Incubate the bottle with the stain at 37 C for 24 hours when it will be ready for use

(A) Staining with Leishman s Wright s or Jenner s stain

Reagents a d appa atus required

(i) Prepared stain preferably in a drop bottle
(ii) Distilled water (pH 7 0) or buffer solution with pH of 6 4 and made with acid monopotassium phosphate and disodium phosphate†
( ) Fresh distilled water which is s ightly acid in reaction
(iv) Staining rack This can be made with plasticine and a pair of glass rod of equal size and thickness and may be placed at one end of a sink away from th water tap or on a rectangular enamell d tray placed near a tap

Glass beakers cylinders a pair of forceps capillary pipettes teats etc

Technique—Put the slides on the staining rack taking care that the side with the blood film is upwards also see that the two ends of the slides are in the same plane

From a drop bottle or with a pipette pour on sufficient stain to cover the whole of the film wait for one minute to allow for proper fixing with a capillary pipette now add distilled water (pH 6 8 to 7 0) or the buffer solution—the same volume in the case of Leishman s and Wrights stains but double the volume in the ca e of Jenner s stains With a capillary pipette or gla s rod thoroughly mix the stain with the diluent to ensure a uniform mixture over the film

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* George T Gurr 136 New Kings Road London S W 6 England

† Monopotassium phosphate	6 63 gm
Anhydrous disodium phosphat	2 56 gm
Distilled water	up to 1 litre

Add 1 c cm of choloroform as preservative

When the mixture is allowed to settle a scum will form on the top if the proportion of the stain and diluent has been correct According to the depth of staining required allow the diluted stain to act for 5 to 10 minutes in the case of Leishman s and Wright s stains and 3 to 5 minutes in the case of Jenner s stain

The diluted or undiluted stains on the slides must not be allowed to dry up at any stage of the staining Drying is prevented by covering the staining rack with a wide bell jar or other improvised device*

When the staining is complete hold one end of the slide firmly with a pair of forceps and place the stain flooded slide under a running tap This will wash off all the stain from the upper surface while the bottom is cleaned by rubbing it well with the fingers of the left hand The slide is now transferred to the beaker containing fresh distilled water and gently shaken to and fro until the colour of the smear becomes faintly pink Now take it out of the beaker wash again under the tap and allow it to dry In order to dry it without allowing dust to adhere to the stained surface the slide should be sloped against a vertical surface e g a wall or the side of a box with the film side inwards

When it is dry the slide is ready to be examined

(B) Staining with Giemsa s stain

Note —It is more difficult to prepare this stain and it is better to purchase it in solution Giemsa s stain as prepared by Gurr is very satisfactory

In staining with Giemsa s stain preliminary fixing with methyl alcohol or some other fixing stain is absolutely necessary

Preparing dilute solution

Take about 20 c cm of prepared distilled water (pH 7 0) or buffer solution in a clean transparent glass cylinder add 20 drops of undiluted stain or in other words as *many drops of stain as there are cubic centimetres of water* Mix well by inverting the cylinder and see that the depth of colour of the mixture is such that when held in front of the eyes it allows a distant object to be seen through it

(*i*) Place the slides to be stained on a staining rack flood the slide with methyl alcohol and cover with a bell jar so that the methyl alcohol does not dry up on the slide Allow the methyl alcohol to act for about 2 minutes remove the bell jar and thoroughly wash the slide with distilled water

Now flood the slide with the diluted stain cover with a bell jar and allow the stain to act overnight Next morning wash and dry the slide as in A

* The precaution *will seldom be necessary* in humid climates e g of Bengal and Assam except in the hottest months but will be imperative in drier provinces in India and in other dry countries such as Iraq Egypt etc

(ii) *Combined staining with Leishman s Wright s or Jenner s and Giemsa s* — This is done exactly in the same way as in A but substituting very dilute Giemsa s stain (1 drop in 2 c cm) as the diluent in place of distilled water or buffer solution

(iii) *Combined staining with May Grunwald* and Giemsa* —Pour on undiluted May Grunwald s stain just sufficient to cover the slide place a bell jar over the staining rack allow the stain to act for 2 to 3 minutes remove the bell jar and add an equal amount of neutral distilled water (pH 7) Allow the diluted stain to act for 5 to 10 minutes according to the depth of staining desired wash thoroughly with neutral distilled water and then flood the slide with dilute Giemsa s stain to act for 15 to 30 minutes according to the depth of the staining desired and wash and dry as in A

Methods used in our laboratory

For all ordinary work Wright s staining method is used and for special work e g examining smears from the marrow counting the nuclear lobes in Arneth and Schilling counts combined May Grunwald and Giemsa s staining are preferred For with the combined May Grunwald and Giemsa s staining all the component parts of both mature and immature red and white cells are very well shown

10
WHITE CELL DIFFERENTIAL COUNT

When making a white cell differential count on slides one should attempt to fulfil the following requirements —

(i) The whole drop of blood should be utilized in making the smear as if only a portion of a large drop is used this portion may not contain a fair sample of the cells (e g the large cells tend to sink to the bottom of the drop)

(ii) The film should be uniformly spread and should be neither too thin nor too thick—the ideal film is one where the margins of the red cells when seen under the microscope just touch one another without overlapping (*vide p 43*)

(iii) The film should occupy the middle two thirds of the slide

(iv) There should be no tails at the end of the film

(v) The cells should be stained well and there should be no debris in between or on the cells

A well stained film shows red cells—an orange buff colour lymphocytes—with pale blue cytoplasm and neutrophil granules—a dull lilac

* This can be purchased in solution and is available from G T Gurr (u s)

Number of cells to be counted

This will vary directly with the total white cell count the higher the total count the larger is the number of the cells that should be counted to get a correct proportion of the different cells The following rule may be taken as a guide but need not be followed rigidly —

Count 100 cells when the total white count is below 5 000 per c mm
Count 200 cells when the total white count is above 5 000 per c mm but below 10 000 per c mm
Count 300 cells when the total white count is above 10 000 per c mm but below 20 000 per c mm
Count 400 cells when the total white count is above 20 000 per c mm

The differential count should be done with an oil immersion lens (one twelfth) and a × 5 eyepiece with a × 10 eyepiece at hand This magnification will enable one to see the details of the cells distinctly but a higher magnification given by the a × 10 eyepiece may sometimes seem desirable e g in the case of doubt arising as to the true identity of a cell In actual practice it will be found that one seldom changes the eyepiece

The microscope must be fitted with a mechanical stage that allows easy movement in either the horizontal or the vertical plane Northern daylight is preferable to artificial light as it shows the true colour of the different cells If artificial light has to be used a blue filter should be placed in front of the light itself or under the condenser of the microscope

Procedure —Place the stained blood film on the mechanical stage of the microscope and fix it securely with the low power (two thirds objective) make a rapid survey of the different parts of the film to find out if the requirements mentioned above are fulfilled If not it is recommended that another properly made film should be stained for to attempt to make a differential count on an unsatisfactory blood film is laborious and irritating and the result obtained is never accurate

If the blood film is satisfactory put a drop of cedar wood oil on the smear rotate the oil immersion lens into position open the diaphragm fully and raise the condenser to get the maximum amount of light With the coarse adjustment lower the oil immersion lens just to touch the stained film through the cedar wood oil then looking into the microscope gently raise the lens until the cells are seen and finally manipulate the fine adjustment until the focus is accurate

The distribution of the white cells in the different portions of the blood films depends on their size the larger and heavier cells e g neutrophils eosinophils and large mononuclears are mostly found along the edges and at the tail end of a smear while the smaller and the lighter cells e g the lymphocytes tend to occupy the middle of the film

Therefore in order to get an absolutely correct differential count every cell in the blood smear on the slide or on both the coverslips used in making the smear

if the coverslip method is used must be counted and classified and the percentage calculated This takes a long time particularly if the smear is a large one it is therefore advisable to make as small a smear as possible

A smear is made from a small drop of blood and one of the methods suggested below is adopted From neither of these methods will the error be very great



Counting the cells

Method A (*This is the method of choice*) Start counting at a point A near the proximal end of the smear and along one edge moving the slide longitudinally counting and classifying the cells all the time up to B which is beyond the end of the film move the slide the breadth of one or two fields to B and again move back along the whole length of the film to C which is beyond the proximal end of the film move across to C and then back again up and down the film Continue to do this until about the middle of the film is reached or until a sufficient number of cells has been counted but always end the counting at the end of the film (*vide* figure 15)

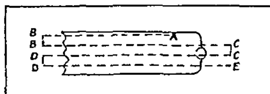


FIG 15

By this procedure you will have included one edge and one or two rows of fields from the rest of the film The majority of the heavy cells are drawn to the distal end and only a few to the sides so that the discrepancy will not be great if after counting one edge you count a little more or a little less of the rest of the film On the other hand it is important to finish at the end so that you will have covered an even proportion of body and tail of the film For this reason an exact figure should not be aimed at but the point of stopping like the point of starting should be at one end of the film The percentages of the different cells are then calculated

Example —Suppose that in making a differential white cell count of a blood film there are 141 neutrophils 63 lymphocytes 12 monocytes and 9 eosinophils i e 225 cells altogether the percentage of the different cells are calculated as follows —

Neutrophils $= \frac{141 \times 100}{225} = 62\ 6$ or 62 7 per cent

Lymphocytes $= \frac{63 \times 100}{225} = 28\ 0$

Monocytes $= \frac{12 \times 100}{225} = 5\ 3$ or 5 3

Eosinophils $= \frac{9 \times 100}{225} = 4\ 0$

Method B *Four field meander technique* —Though this method is recommended in most textbooks we consider that it allows greater scope for individual variation in procedure and we prefer the previous method the method is as follows —

From a point at the edge of the film move towards the centre of the film for about four fields then using the other mechanical stage adjustment move about four fields towards the proximal end of the film then back towards and beyond the edge move a few fields towards the proximal end and repeat the process until 50 cells have been counted and classified This process should be repeated in four different places at the edge of the film on either side and near each end of the film (*vide* figure 16)

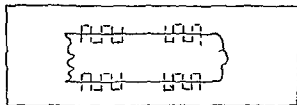


Fig 16

Recording the results

Except in cases of leukæmia and some rare pathological conditions the types of white cell that are usually seen in the differential count of a peripheral blood film are neutrophils lymphocytes monocytes eosinophils and basophils in the order of frequency in which they are encountered As the slide is moved from one field of vision to another the examiner must record both the total number and also the types of cell encountered

This may be done in different ways —

I The usual method is to write down on a piece of paper the names of the cells in the order of frequency in which they usually occur in the peripheral blood and to put a vertical stroke against each name as that cell is seen in the field of vision making the fifth stroke cross the other four so that the strokes are in groups of five and easily counted (*vide* example 1) When all the cells in the film or in the case of a large film in the area decided upon have been counted they are totalled and the percentage calculated

Example 1 —

N — 卌 卌 卌 卌 卌 卌 卌
卌 卌 卌 卌 卌 卌 /// = 68
L — 卌 卌 卌 卌 卌 卌 // = 32
M — 卌 = 5
E — 卌 / = 6
B — / = 1

II Another method is to memorize the numbers of the two main cell types until one of the other types is encountered then to enter the count on your prepared list and begin again (*vide* example 2)

The numbers against each type are added and the percentages calculated

Example 2 —

												Total	Percentage
Neutrophils	10	12	14	17	20	4	10	14	1	20	10	13	= 68 0
Lymphocytes	4	3	5	8	5	1	8	4	1	10	2	51	= 26 3
Monocytes		1	1	1	1		1	1		1		7	= 3 6
Eosinophils	1					1			1			3	= 1 5
Basophils											1	1	= 0 5
												194	

III A quicker method which can be learnt with a little practice is to remember the numbers of the different types of cell in the traditional sequence neutrophil lymphocytes monocytes eosinophil and basophil until 25 cells have been counted the number of each type counted is entered against the respective name on a piece of paper and when the required number of cells have been counted they are totalled and the percentages calculated (*vide* example 3)

Example 3 —

									Total	Percentage
Neutrophils	18	17	10	14	16	14	20	11	120	60 0
Lymphocytes	6	5	12	8	7	9	4	9	60	= 30 0
Monocytes	0	3	2	1	2	1	1	3	13	= 6 5
Eosinophils	1	0	1	1	0	1	0	2	6	= 3 0
Basophil	0	0	0	1	0	0	0	0	1	= 0 5
									200	

Method I is simple and fool proof but slow method II is also simple and saves the repeated interruptions of method I method III is very popular but tends to make one stop at a round number and thereby simplify the calculation which is a procedure not in the best interests of accurate countings

In methods I and II theoretically one has to stop periodically and add up all the figures to see if sufficient cells have been counted but this can be obviated by the use of a mechanical counter e g the Veeder Root hand tally The counter fits nicely into the hollow of the palm and is conveniently kept in the left hand The knob is pressed with the thumb each time that a cell is seen and the numbers are at once recorded on the dial The total number of cells counted at any time during the process of the differential count can be seen by simply looking at the dial of the counter

Expressing the results

It is customary to express the result in the white cell differential count as a percentage of the various cells encountered without any reference to the total white cell count This does not always convey the full significance of the observation and both percentage and absolute figures should be given

The absolute figures are readily obtained by striking out the last two zeros of the total leucocyte count or moving the decimal point back two spaces and multiplying each percentage by this figure as in the example given below —

Example —(A fairly normal count)

Total white cell count—7 500 per c mm

Differential white cell count—

	Per cent	Total per c mm
Neutrophils	68 0	5 100
Lymphocytes	24 0	1 800
Monocytes	5 0	375
Eosinophils	2 5	187
Basophils	0 5	37

Now if the patient were to take some toxic drug which reduced the granulocytes to a low figure say to 540 per c mm but did not affect the other leucocytes the total count would now be 2 700 per c mm and the percentages—

	Per cent	Total per c mm
Neutrophils	19	513
Lymphocytes	67	1 809
Monocytes	13	351
Eosinophils	1	27
Basophils	0	0

In this count the attention is attracted by the high lymphocyte and monocyte percentages whereas actually these cell elements are normal and all that has happened is a decrease in neutrophils

Again in some conditions e g asthma eosinophilic lung idiopathic eosinophilia etc the percentage of eosinophils may be very high considerably reducing the percentage of other cell elements

Example

Total white cell count—25 000 per c mm

Differential white cell count—

	Per cent	Total per c mm
Neutrophils	30	7 500
Lymphocytes	8	2 000
Monocytes	1 5	375
Eosinophils	60	15 000
Basophils	0 5	125

In this example though the high eosinophil count is apparent a wrong impression about the values of neutrophils lymphocytes and monocytes which are of the normal order is liable to be made from the percentage figures only

Therefore a differential count should always be given in absolute figures as well as in percentages

Normal white cell counts —The normal leucocyte count in an adult is usually given as 7 000 to 9 000 per c mm A table (IX) showing the normal white cell counts in different populations is given

TABLE IX

Differential Leucocyte counts in normal individuals

Number	Sex and age	Residence	Neutrophils p c	Neutrophils per c mm	Lymphocytes p c	Lymphocytes per c mm	Monocytes p c	Monocytes per c mm	Eosinophils p c	Eosinophils per c mm	Basophils p c	Basophils per c mm	Authority
50	Male adults	Calcutta					Mean 7 00		5 90				Napier and Das Gupta (unpublished)
							S D ±3 91		±5 19				
114	Female adults	Calcutta	Mean 62 6	4 507	6 9	1 937	5 5	390	4 8	346	0 2	14	Napier Edward and Das Gupta 1944
198	Males 19 39 years	U S A	Avge 53 8	3 9 0	38 8	2 800	4 2	310	2	150	0 5	30	Osgood et al 1939
			Max 86 0	9 850	68 0	4 900	12 0	1 360	10	980	4 0	340	
			Min 22 0	1 000	16 0	800							
71	Females 26 36 years	U S A	Avge 55 8	4 220	36	2 630	4 2	310	1 5	110	0 5	30	
			Max 94 0	11 000	61	6 500	11 0	1 040	9	690	3	720	
			Min 23 0	1 900	6	700							

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50	Male adults	Calcutta						Mean 7 00		5 90				Napier and Das Gupta (unpublished)
								S D ±1 91		±5 19				
114	Female adults	Calcutta	Mean	62 6	4 507	26 9	1 937	5 5	396	4 8	346	0 2	14	Napier Edward and Das Gupta 1944
198	Males 19-38 years	U S A	Avge	53 8	3 9 0	38 8	2 800	4 2	310	2	150	0 5	30	Osgood et al 1939
			Max	86 0	9 850	63 0	4 900	12 0	1 360	10	980	4 0	340	
			Min	22 0	1 000	16 0	800							
71	Females 26-36 years	U S A	Avge	55 8	4 220	36	2 630	4 2	310	1 5	110	0 5	30	
			Max	94 0	11 000	61	6 500	11 0	1 040	9	690	3	720	
			Min	29 0	1 900	6	700							

It will be noted that in Indian the monocyte counts are above the usual normal figure for Europeans and Americans the eosinophil figures are also high compared with European and American standards

10

CELL IDENTIFICATION

The first essential for accurate identification of the cells of the blood or marrow is a satisfactory and properly stained blood film A well stained film should show no precipitate on or between the cells the red cells should stain an orange buff the neutrophil granules a dull shade of lilac and in the monocyte the so-called azurophil granules should just be visible—with any of the Romanowsky stains

The following system is recommended for the identification of different cells

Granules—First ascertain whether the cytoplasm of the cells contains any granules or not If there are granules decide whether the granules are neutrophilic eosinophilic or basophilic

Neutrophil granules are small uniform in size uncountably numerous but discrete and stain a dull shade of lilac

Eosinophil granules are large round uniform in size and stain orange red with pale centres occasionally eosinophil granules take on a bluish stain even in a well stained specimen while in a badly stained one all may be bluish The eosinophil granules are not so numerous nor so discrete as neutrophil granules

Basophil granules vary considerably in size in the same cell, they stain a dark blue violet colour which is entirely different from the colour of the nucleus they are always coarser and fewer in number than the neutrophil or eosinophil granules The granules are always found superimposed on the nucleus as well as in the cytoplasm

Besides these specific granules the presence of which characterizes the cells of the granular series some cells of the non granular series e g monocytes and lymphocytes may show some stained particles in the cytoplasm of the cells which have been called azurophil granules These azurophil particles are of the same colour as the nucleus of the cell in which they occur but may be paler brighter or darker They vary in size in depth of staining and in number In the lymphocytes the particles are few in number they are large coarse and darkly stained and occur in small groups in the cytoplasm while the particles in the monocytes are more numerous paler and finer and are more evenly distributed in the cytoplasm

Having noted whether granules are present or not and if present the nature of the granules employ the following tables for the further identification of the cells

Table X gives the cells of the non granular series and Table XI those of the granular series

The cells of the granular series all originate from the primary differentiated cell of that series the myeloblast They are arranged in the table in order of maturation

The mature neutrophil eosinophil and basophil have their specific precursors in the earlier cells of the series and these can be distinguished by the size and colour of their granules (*v s*) there are thus neutrophil eosinophil and basophil of each developmental stage pre myelocytes meta myelocytes and staff cells

Table XII is a supplementary table to aid in the identification of primary differentiated cells which are already shown in Table X

The points of difference in the morphology of the different primary cells are very fine and at times it is almost impossible to distinguish one from the other In cases of leukæmia or anæmia the identification of an individual cell has often to be made not on the cell alone but on the characteristics displayed by other associated cells in other words the cell is judged by the company it keeps

Table XIII is also supplementary It gives the nucleated cells of the red cell series

The development of blood cells

We do not propose to discuss the origin of the cells found in the blood stream for it is a controversial field in which unitarians dualists and trialists of the monophyletic and polyphyletic school argue with one another to the confusion of the practical hæmatologist We are only concerned with the cells already differentiated the immediate precursors of the normal and abnormal cells of the peripheral circulation

It is however impossible to avoid controversy altogether as this is intimately associated with nomenclature which is definitely our domain for at present much confusion in the literature is due to different writers adopting different names for the same cell and the same name for different cells

We have followed Israel (1939) in using the term Pro-erythroblast for the earliest differentiated percursor of both normal and abnormal red cells and have used the expression megaloblast for the cell that are encountered in Addison s pernicious anæmia and in other macrocytic anæmias

With reference to the other cells we have taken the view that the granulocytes lymphocytes monocytes plasma cell and platelets have each a separate differentiated precursor cell and following the usual practice we have called these myelo blast lymphoblast monoblast plasmablast and megakaryocyte respectively

It will be noted that in Indian the monocyte counts are above the usual normal figure for Europeans and Americans the eosinophil figures are also high compared with European and American standards

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TABLE XI

Identification of cells containing granules

Nucleoli	Nucleus	Granules	Cytoplasm	Cell	Mark	REMARKS
Present	Round or oval relatively fine chromatin structure	*Very few* fine scattered	Pale blue Relatively small amount	Pre myelocyte	*Ia*	This as well as the other granular cells *may* be *neutrophil* eosinophil or basophil Basophil pre myelocytes are rarely identified
Generally absent	Round or oval coarse chromatin structure may not be very well stained	Very prominent coarse	Very pale blue variable in amount but usually more than in *Ia* not well seen as it is covered by granules	Myelocyte	*Ib*	In the eosinophil and basophil myelocytes the cytoplasm is almost entirely covered by granules and is rarely visible In the basophil the nucleus also is blurred by granules
Absent	*Bean* or *kidney* shaped coarse chromatin structure	Granules less prominent than corresponding granules in *Ib* numerous	Large in amount Very pale blue obscured by granules	Meta myelocyte	*Ic*	The basophil meta myelocyte and staff cells are very difficult to identify
	Curved rod coarse chromatin structure	*Finer and more* numerous than corresponding granules in *Ic*	*Large amount* Very pale blue	Staff (or band)	*Id*	
	Lobed or segmented 2 to 5 or *more lobes*	Very fine and numerous light lilac *colour*	Large amount Very light greyish blue	Neutrophil *polymorphonuclear* granulocyte	*Ie* (i)	
	Usually bilobed rarely may have more lobes	Finer than eosinophilic precursor *Id* (ii) but coarser than *Ie* (i)	Very pale blue scarcely seen	Eosinophil granulocyte	*Ie* (ii)	
	Segmentation of the lobes is difficult *to make out* but never more than two lobes	Very coarse dark violet blue almost *black often obscuring* nucleus	Very pale blue scarcely seen	Basophil granulocyte	*Ie* (iii)	

TABLE X

Identification of cells without specific granules

Nucleoli	Nucleus	Chromatin Structure	*Cytoplasm*	Size of nucleus in relation to cell	Azurophil particles	Name of cell	Identification mark
	Round, oval or irregular	Fine	Light blue clear	More than three quarters		Myeloblast	I
	Round or oval	Finer than I	Deeper blue than I clear			Lymphoblast	II
Present	Round, oval or irregular	Relatively coarse	Light blue opaque	More than two thirds	None	Monoblast	III
	Round or oval	Fine stippled	Light grey blue opaque	More than three quarters		Proerythroblast	IV
	Round or oval	Fine but not so fine as I and III	Deep blue opaque	More than half		Plasmablast	V
Present or absent	Kidney shaped or irregular	Coarser than III	Light blue opaque		May be present	Premonocyte	III (*a*)
	Round or oval	Coarse	Light blue clear		May be present	Large Lymphocyte	II (*a*)
	Generally round or oval *may* be clover leaf	Coarse in clumps	Clear transparent blue may appear as a thin rim in smaller cells	More than half in larger cells but almost fills the cells in smaller ones	May be present few unevenly distributed	Lymphocyte	II (*b*)
	Horse shoe or irregular	Coarse clumps or strands	Light faded blue opaque	More than half	Usually present very fine numerous scattered	Monocyte	III (*b*)
	Round or oval	Coarse	Basophils	More than two thirds		Normoblast A	IV (*ai*)
	Round or oval	Coarser than (IV(ai))	Basophilic Polychromatic or slightly eosinophilic	Not more than half		Normoblast B	IV (*aii*)
	Round or irregular	Pycnotic	Polychromatic or eosinophilic	Variable		Normoblast C	IV (*aiii*)
	Round or oval	Fine characteristic open net work	Dark Grey blue opaque perinuclear pale area may *be present*	More than two third		Megaloblast A	IV (*bi*)
	Round or oval	Network is still more open than IV(bi)	Basophilic polychromatic or slightly eosinophilic	Less than two thirds	None	Megaloblast B	IV (*bii*)
	Round, oval or irregular	Indefinite	Polychromatic or Eosinophilic	*Less than half*		Megaloblast C	IV (*biii*)
	Round or oval	Coarse	Deep blue opaque with a perinuclear pale area	Less than half		Preplasmacyte	V (*a*)
	Round or oval	Very coarse cart wheel appearance	Deep Greyish blue ground glass	Less than half *eccentric*		*Plasma cell*	V (*b*)
	Irregular and Multi lobed	Coarse	Light blue	Variable	Present fine numerous	Megakaryocyte*	VI

Mature platelets (VIa) which develop from megakaryocyte are small (2 3μ) ill defined structures not clearly differentiated into nucleus and cytoplasm

TABLE XIII

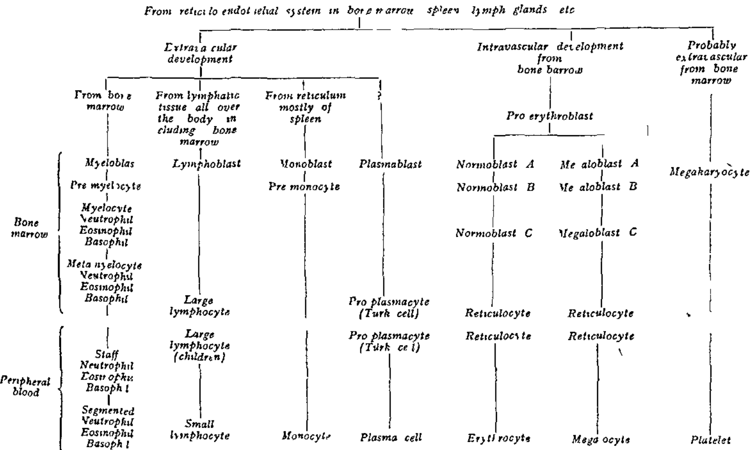
Identification of the diffrent nucleated cells of red cell series

Cell	Size	Cell outline	Cytoplasm	Nucleus	Chromatin	Nucleoli
Pro-erythroblast	16-21μ	Irregular	Light grey blue opaque (basophilic)	Large occupies most of the cell	Fine stippled stains lightly resembles a mass of unravelled yarn	Present few appear as irregular gaps
Normoblast A	11-17μ	Regular	Bluish grey opaque	Large occupies over two thirds of the cell	Coarse arranged rather half hazard in lumpy dark staining masses with light areas between	Absent
Normoblast B	8-14μ	Regular	Basophilic polychromatic or eosinophilic	Occupies about half of the cell	Coarser than in normoblast A seen in lumps occasionally has a cart wheel appearance	Absent
Normoblast C	6-10μ	Regular	Polychromatic or eosinophilic	Sometimes eccentric relative size variable	No definite structure Pycnotic appears as a drop of ink	Absent
Megaloblast A	14-21μ	Irregular	Dark grey blue opaque generally with a pale area around the nucleus	Large occupies more than two thirds of the cell	Fine has a characteristic open network	Absent
Megaloblast B	2 0μ	Slightly irregular	Basophilic or polychromatic	Large but smaller than in megaloblast A	The characteristic open network is still more open than in megaloblast A	Absent
Megaloblast C	9-15	Regular	Polychromatic or eosinophilic	Small stains darkly and generally eccentric	No very definite pattern but still may have some resemblance to the open network arrangement of the nucleus of the megaloblast series	Absent

TABLE XII

Identification of the earliest differentiated cells

Name of cell	Nucleoli	Nucleus	Chromatin	Nuclear membrane	Cytoplasm	Auer's bodies
I Myeloblast	Few, not very distinct	Round or oval usually central	Fine reticulation	Not distinct and no condensation of chromatin at edges of nucleus	Clear light transparent blue	May be seen occasionally
II Lymphoblast	Many very distinct	Round or oval and central	Finer reticulation	Distinct with condensation of chromatin at the edges	Clear deeper blue	Not seen
III Monoblast	One or two often clean cut	Round or oval often folded	Relatively coarse	Not distinct and no condensation of chromatin at edge	Light blue not so transparent	Frequently seen
IV Pro-erythroblast	Few appear as irregular gaps	Round or oval	Fine stippled	Not distinct and no condensation of chromatin at edges	Light grey blue ground glass	None
V Plasmablast	Few, not distinct	Round or oval	Fine	Not distinct	Deep blue opaque	None


SCHEMA SHOWING ORIGIN OF BLOOD CELLS
From reticulo endothelial system in bone marrow spleen lymph glands etc
Extravascular development
Intravascular development from bone barrow
Probably extravascular from bone marrow
From bone marrow
From lymphatic tissue all over the body including bone marrow
From reticulum mostly of spleen
?
Pro erythroblast
Bone marrow
Myeloblas
Pre myelocyte
Myelocyte Neutrophil Eosinophil Basophil
Meta myelocyte Neutrophil Eosinophil Basophil
Lymphoblast
Large lymphocyte
Monoblast
Pre monocyte
Plasmablast
Pro plasmacyte (Turk cell)
Normoblast A
Normoblast B
Normoblast C
Megaloblast A
Megaloblast B
Megaloblast C
Megakaryocyte
Reticulocyte
Peripheral blood
Staff Neutrophil Eosinophil Basophil
Segmented Neutrophil Eosinophil Basophil
Large lymphocyte (children)
Small lymphocyte
Monocyte
Plasma cell
Erythrocyte
Megalocyte
Platelet

Explanation of Plate No I

I *Myeloblasts* (Nucleoli have not been reproduc d)

Ia *Premyelocytes* showing fine granules the fou th cell an eo inophil precurso is slightly more mature than the rest

Ib *Myelocytes* the cells in the upper are less mature than those in the lower row the first three in each row are neutrophils and the la t two eos noph ls

Ic *Met myelocytes* the la t two (Ic i) are eo inophils

Id *Staff or band forms* the last two are eosinophils (I l) (Tl e granules n tl last cell have not been well reproduced)

Ie *Mature granulocytes* the first three are neutroph ls and tl e last t o eo inophil

Ib(iii) Ic(i i) and Ie(i) *Basopl ils* myelocyte m ta myel yte and matur The shape of the nu leus is the only feature on which the immature form are identifie l In the original drawing the shapes of the nuclei were just dis ernible but in the reproduction they are obs ured

III *Monoblasts* the lower two sho ving Auer s bod es

IIIa *Premonocytes*

IIIb *Monocytes* the se on l cell in the top row might be cla ed s a premonocyt but tl e azure particles are well develop e l (There l ould l e more blue colour n the cytopla m of tl ese cells)

VI *Megakaryocyte* these cells are often proportionately mu h b gger than the on shown

VIa Platelets

Magnification about $\times$ 1 000

Sta ning—May G un ald and G emsa

I

Ia

I a(I) Ia(II)

Ib

Ib (II)

Ic

Ic(I) I c(II)

Id

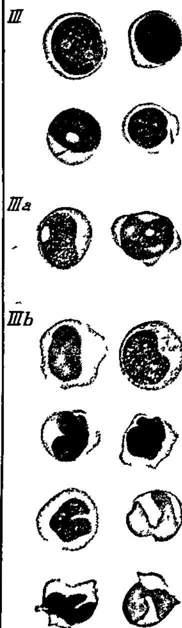
I d(I) I d(II)

Ie

I e(I) I e(II)

Ib (III) Ic(III) Ie (III)

H Roy 40





VIa

II





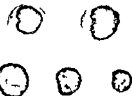


IIa





IIb





V



Explanation of Plate No III

II *Lymphoblasts*

IIa *Large lymphocytes* with azure particles

IIb Mature (small) *lymphocytes*, the first with azure particles

V (above) *[illegible]* (Türk's cell)

V (below) *Plasma cells*

(The characteristic structure of the nucleus in the cells of the plasma cell series is not very clear in any of the cells. There should be more blue colour in the cytoplasm of these cells.)

Left lower corner: Cresyl blue staining

Erythrocytes, reticulocytes, platelets

Magnification about × 1000

Staining—May Grünwald and Giemsa, except the cells in the left lower corner in which all the cells are stained with cresyl blue (see p. 4 method B)



PLATE No II



IV

IVa i      IVb i

IVa ii      IVb ii

IVa iii       IVb iii

IVc      

IVd     

I II III IV

Explanation of Plate No II

IV *Pro-erythroblast*
IVa i *Normoblast* A (first three cells)
IVa ii *Normoblast* B (first three cells)
IVa iii *Normoblast* C (first four cells)
IVb i *Megaloblast* A (last two cells)
IVb ii *Megaloblast* B
IVb iii *Megaloblast* C ,,
IVc *Reticulocytes*

IVd Abnormal Erythrocyte
(i) *Red cell showing Howell Jolly bodies*
(ii) *Red cell showing Cabot's rings*
(iii) *Poikilocyte*
(iv) *Red cell with stippling*

Staining—May Grunwald and Giemsa except figures in row IVc which are stained first with cresyl blue and then with Wright's stain [Vide p 23 method B]

Red cells.—Normally the red cells originate intravascularly from the endothelial cells of the sinusoidal spaces and capillaries of the bone marrow. The first differentiated cell is the pro-erythroblast. As development proceeds the pro-erythroblast passes through the different stages of normoblast and reticulocyte to develop into the mature erythrocyte (*vide* schema, p. 60). In the process of maturation the nucleus loses its nucleoli very early so that these are not seen in the normoblast A; the nucleus also loses its reticular structure and becomes gradually coarser and coarser as development proceeds until in the normoblast C it becomes completely pycnotic; after this the nucleus is lost (by fragmentation or extrusion) and the erythrocyte becomes fully mature. The reticulocyte is a stage between the normoblast C and the fully mature red cell but the reticulations are not seen in a Romanowsky stained film.

In pathological condition as in cases of macrocytic anæmias some of the pro-erythroblasts instead of developing into normoblasts develop into megaloblasts which passing through the different stages mature into megalocytes. Nucleoli are not seen even in the earliest megaloblasts megaloblast A. In the earlier cells megaloblast A and megaloblast B the nucleus has a characteristic open network of the chromatin which is not seen in the later stages megaloblast C.

Granulocytes.—The granular cells develop extravascularly from the reticulo-endothelial cell of the bone marrow. The earliest differentiated cell is the myeloblast.

The first evidence of maturation of the myeloblast is the appearance of granules in the cytoplasm. At first the granules are very fine and few as in the pre-myelocytes but gradually they become more numerous and coarser and are coarsest at the myelocytic stage after which the granules again become finer and less numerous as development proceeds. In the fully developed neutrophil polymorphonuclear cells the granules are very fine and numerous. The cells can be differentiated as neutrophil, eosinophil and basophil according to the colour and size of their respective granules with difficulty at the pre-myelocyte stage but quite easily from the myelocyte stage onward.

In the process of development the nucleus loses its nucleoli; these are ill defined in the pre-myelocyte and are almost always absent from the myelocyte stage onwards. The chromatin structure of the nucleus which is finely reticular in the myeloblast becomes coarser and more condensed as development proceeds and is very dense at the later stages; this condensation makes the nucleus smaller. The shape of the nucleus too changes from round or oval at the earlier myeloblast, pre-myelocyte and myelocyte stages to kidney or bean shape at the meta-myelocyte stage and then it becomes elongated in the staff and finally lobulated in the segmented forms.

The cytoplasm which is blue and scanty in the myeloblast loses its colour gradually and becomes more abundant as development proceeds—in the later stages the cytoplasm is hardly visible as it is covered by the granules.

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Granulocytes.—The granular cells develop extravascularly from the reticulo-endothelial cells of the bone marrow. The earliest differentiated cell is the myeloblast.

The first evidence of maturation of the myeloblast is the appearance of granules in the cytoplasm. At first the granules are very fine and few as in the pre-myelocytes but gradually they become more numerous and coarser and are coarsest at the myelocytic stage after which the granules again become finer and less numerous as development proceeds. In the fully developed neutrophil polymorphonuclear cells the granules are very fine and numerous. The cells can be differentiated as neutrophil, eosinophil and basophil according to the colour and size of their respective granules with difficulty at the pre-myelocyte stage but quite easily from the myelocyte stage onwards.

In the process of development the nucleus loses its nucleoli; these are ill defined in the pre-myelocyte and are almost always absent from the myelocyte stage onwards. The chromatin structure of the nucleus which is finely reticular in the myeloblast becomes coarser and more condensed as development proceeds and is very dense at the later stages; this condensation makes the nucleus smaller. The shape of the nucleus too changes from round or oval at the earlier myeloblast, pre-myelocyte and myelocyte stages to kidney or bean shape at the meta-myelocyte stage and then it becomes elongated in the staff and finally lobulated in the segmented forms.

The cytoplasm which is blue and scanty in the myeloblast loses its colour gradually and becomes more abundant as development proceeds—in the later stages the cytoplasm is hardly visible as it is covered by the granules.

Monocytes.—The monocytes originate from the reticulum all over the body but particularly from that in the spleen The first differentiated cell is the monoblast it proceeds through the stag of the pre monocyte to the stage of fully developed monocyte As development proceeds the nucleus loses its nucleoli and the fine reticular structure becomes coarser and very fine and numerous azure particles appear in the cytoplasm of a fully developed monocyte The nucleus which is round or oval in the monobla t becomes slightly indented in the pre monocyte and may take on various shapes in the mature monocytes (We have made no attempt to differentiate between monocytes and histiocytes this can only be done by a supra vital staining process which we are not describing here Some of the large mononuclear cells in the peripheral blood are undoubtedly histiocytes who e origin is probably different from that of the monocytes)

Lymphocytes

The lymphocytes originate mainly from the lymphatic tissue of the lymph glands all over the body and to a small extent from the lymphoid tissue in the marrow The first differentiated cell is the lymphoblast which proceeds through the stage of large lymphocyte to the fully developed (small) lymphocyte

In the process of development the nucleus loses the nucleoli the fine chromatin structure becomes coarser and finally in the mature cells it is pycnotic The cytoplasm which is definitely blue in the lymphoblast becomes lighter in colour as development proceeds but is always transparent In some of the cells a few coarse irregularly scattered azure particles may be seen

12
TECHNIQUE OF STERNAL PUNCTURE

Bone puncture as an aid to accurate diagnosis of blood diseases and to the study of their ætiology has only come into general use during the last few years the main reason for this was that the methods used for obtaining material from the bones were comparatively difficult and usually painful unless anæsthetic was given before the Salah sternal puncture needle was introduced This handy and inexpensive instrument is now used widely not only in hæmatological work but in the diagnosis of kala azar and other protozoal and bacterial disea es We have used this needle for about four years and have adopted sternal puncture as a routine procedure in all cases of anæmia

The senior writer described this technique in a paper written in co operation with Dr P C Sen Gupta* (Napier and Sen Gupta 1938) since then as a result of further experience certain modifications in technique have been introduced and these are incorporated in this description but the technique described is materially the same as that given in the above mentioned paper

* With whose kind permission the figures from this paper have been reproduced

Apparatus required

(i) The sternal puncture needle
(ii) Two Record syringes 2 c cm
(iii) A pair of scissors and a shaving set
(iv) Two per cent solution of novocaine or any of its substitutes (Pitument 4 per cent solution produces good anæsthesia)
(v) Absolute alcohol and ether
(vi) Collodion or tincture of benzoin
(vii) Cotton wool etc

The needle is dry sterilized in a hot air sterilizer in a test tube The syringe is sterilized by boiling and then dried thoroughly by first driving out water with alcohol and later alcohol with ether and finally by drawing in and expelling hot air (through the flame of a gas burner or spirit lamp)

The sternal puncture needle

The Salah needle used for sternal puncture is shown (figure 17) It is made of rustless steel and the bore is about the same as that of a lumbar puncture needle The guard C on the needle can be moved so as to adjust the depth of the puncture Usually the guard has to be fixed at a distance of 1 to 1 5 cm from the tip in order that the marrow may be reached

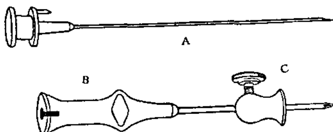


FIG 17—Needle B with stylet A removable movable guard C (actual size)

This distance will vary with the thickness of the skin and subcutaneous tissue of the thoracic wall in fat individuals as much as 2 cm may be required and in very emaciated ones less than 1 cm It may be found advisable to readjust the guard after the needle has reached the periosteum before it is pushed through the outer plate into the marrow cavity The stylet A is kept in while the puncture is being made and is withdrawn after the cavity is reached

Procedure

The hair over the sternum if there is any is first clipped with a pair of scissors shaved with a razor and the skin finally cleaned thoroughly with alcohol The best site for the puncture is just to one side of the middle line at the level of the second intercostal space This area is first anæsthetized by infiltration with a 2 per cent solution of novocaine or its substitute

Some solution is first injected into the skin with a fine needle attached to a 2 c cm syringe then the needle is pushed down to the periosteum and the rest of



FIG 18.—*Needle in position after the marrow cavity has been penetrated*

the solution injected About 1 c cm is usually sufficient in a thin individual but more is *required where the subcutaneous tissue is deeper After an interval of 5* to 10 minutes the actual puncture is made

The apparatus is held with the knob of the stylet in the palm of the hand and the needle itself between the thumb and index finger the latter on the guard C of the needle Pressure is applied and the skin and subcutaneous tissues are pierced a rotatory movement will then facilitate puncture of the outer plate of the sternum (figure 18) As the external plate of the sternum is pierced and the marrow cavity is *entered there is a sensation of loss of resistance just as is felt on entering the spinal* canal during lumbar puncture The stylet is now taken out a 2-c cm Record syringe is attached to the end of the needle and the marrow blood is aspirated When the fluid is aspirated the patient feels a dragging pain (figure 19) which is a guide as to whether the needle is in the marrow cavity or not About 0 5 c cm of marrow (sinusoidal) blood is *removed and the syringe and the sternal puncture* needle are withdrawn digital pressure is applied over the puncture for a minute *or two* and the puncture is sealed with collodion The needle is detached from the syringe and the latter is inverted several times so as to mix the contents thoroughly then small drops are placed on clean slides and smears are made

(*vide* p 43) The rest of the fluid is put into an oxalate tube for total nucleated cell count

Only very rarely will one fail to obtain blood The commonest error is to fail to allow a sufficient length of needle In this case the guard must be adjusted slightly the stylet replaced and the needle pushed in a little deeper Occasionally the needle goes too deeply and has to be withdrawn slightly before blood will come Our only complete failure was in a case of leukæmia and in none of our cases of leukæmia has the blood come freely as the marrow is packed with cells in such cases

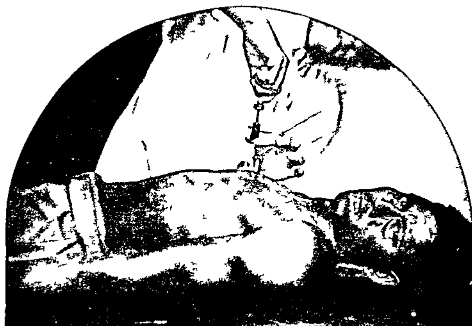


FIG 19.—The stylet has been removed and sinusoidal blood is being drawn into a Record syringe usually the only painful part of the operation

Not more than 0 5 cm of blood is aspirated because if more is drawn there is a probability that the negative pressure in the marrow cavity will draw blood from the vessels in the locality and thus dilute the sinusoidal blood It is probably impossible entirely to prevent this occurring therefore a constant amount of blood is drawn to obviate gross differences in the degree of dilution in the samples taken from different persons The syringe is inverted several times in order that the contents may be mixed thoroughly This is necessary because the fluid that comes out at the beginning is not the same as regards cellular content as the fluid that comes out towards the end of the aspiration *

* Vide *Ann Rep Calcutta School of Trop Med for* 1938

Examination of material

(1) Examination of the stained smears —

The sternal puncture smears are best stained with combined May Grunwald and Giemsa staining this shows the nuclear details very clearly Failing this satisfactory results for ordinary work may be obtained by staining with Leishman s or Wright s stain (*vide* p 45)

An accurate differential count of the different nucleated cells is made by counting 500 or more nucleated cells from different parts of a well stained smear The criteria on which the different cells are identified are given above

(2) The oxalated specimen is examined *for enumeration of total nucleated* cells (*vide* p 20)

(3) The oxalated specimen may also be examined for estimation of hæmoglobin (*vide* p 3), enumeration of red cell (*vide* p 13) and estimation o reticulocyte percentage (*vide* p 21) if the informations are required for any special purpose

Discussion

The material which is obtained by this procedure is neither peripheral blood (obviously) nor bone marrow but is blood from both the patent and the closed sinusoidal spaces in the hæmopoietic tissue of the bone marrow, in which are mixed a few cells detached from the walls of these sinuses by the intruding needle or by the act of aspiration The extent to which the detached tissue cells are added to the sinusoidal blood probably varies with each puncture and constitutes th weakness of the procedure from the point of view of obtaining a true and unvarying picture of bone marrow hæmopoietic activity However the extreme variations *in a single subject that have been reported* by some workers and the differences in the normals given by different workers can probably to some extent be accounted for by variations or defects in the technique employed e g by the failure to remove a small and constant amount of blood by failure to mix the blood before making smears or by making counts from one part of the blood film only

Zanaty (1937) has pointed out that wide variations may exist in the total nucleated cell counts and that from these counts little idea of the activity of the bone marrow can be obtained but from our experience of sternal puncture we believe that there is *generally* a distinct *correlation between hæmopoietic* activity and the total nucleated cell count though this may on occasions be misleading

Both the hæmoglobin and red cell count are slightly lower and reticulocyte count slightly higher in the sinusoidal blood than in the venous blood but these estimations are of little practical value

Normal standards

It is useful to know what proportions of the various cells one may expect o find in a sternal puncture The data given by various writers are *difficult to* correlate because they have adopted different methods of classification and different nomenclatures and we prefer to quote only our own findings

The data given in Tables XIV and XV are from two sources —

(*a*) First series 10 normal individuals males 2 c cm of fluid taken

(*b*) Second series 53 subjects infected with filariasis 0 5 c cm of fluid taken

We have quoted the latter series because we believe that they represent the findings in normal individuals very close y and because it is not easy to persuade 50 perfectly healthy individual to undergo a sternal puncture One might expect them to differ from the normal in the matter of the eosinophil cells but in actual fact the percentage is lower than in the first normal series The material differences that will be noted between the two series are almost certainly due mainly to the fact that in the second series we took a smaller amount of fluid and that there was consequently less dilution by systemic blood

The main discrepancies are the higher total nucleated cell count in the second series—higher than the normal figure usually quoted a lower myeloid/nucleated red-cell ratio—also lower than the usual normal figure a higher lymphocyte percentage and a higher maximum figure for most of the cells—which may be due to the larger number of subjects involved in the second series

For the findings of other workers reference should be made to Zanaty (*loc cit*) and Scott (1939)

TABLE XIV

Sternal puncture normal data

	First series		Second series	
	Mean	Standard deviation	Mean	Standard deviation
Hæmoglobin in grammes per 100 c cm	13 43	± 0 93		
Red cells per c mm in millions	4 99	± 0 40		
Reticulocytes—percentage of red cells	0 75	± 0 39		
Total nucleated cells per c mm	53 500	± 26 500	111 678	± 64 832
Nucleated red cells—percentage of total nucleated cells	25 75	± 4 07	27 606	± 11 746
Leucocytes—percentage of total nucleated cells	74 25	± 4 07	72 4	
Granulocytes—percentage of total nucleated cells	64 40	± 5 10	51 529	± 11 421
Non granular leucocytes—percentage of total leucocytes	9·85	± 2 43	17 889	±6 796
Myeloid/nucleated red-cell ratio	2 50		1 975	

We seldom identify cells as lymphoblasts or monoblasts except in cases of leukæmia but myeloblasts are occasionally found and appear in most of our

TABLE XV

Sternal puncture differential nucleated cell count

		Series I		Series II		
		Range	Mean	Range	Mean	Standard deviation
Nucleated red cells	Megaloblasts	0 0 to 1 5	0 7	0 0 to 3 25	0 92	± 0 7366
	Erythroblasts	0 4 to 10 0 (Erythroblasts and Macroblasts)	3 4	0 0 to 3 3	0 49	± 0 6619
	Macroblasts			0 0 to 8 75	2 075	± 1 7675
	Normoblast	16 0 to 25 5	21 6	7 5 to 63 2	24 17	± 10 4505
White cell series	Myeloblasts	0 4 to 1 5	1 2	0 0 to 0 75	0 15	± 0 2670
A Granular series	Pre myelocytes	0 0 to 1 5	0 7	0 0 to 2 0	0 44	± 0 4409
	Myelocytes—					
	Neutrophil	1 0 to 9 5	4 4	1 0 to 18 75	8 30	± 3 2061
	Eosinophil	0 4 to 2 6	1 3	0 0 to 4 0	1 445	± 0 9498
	Basophil	None found		None found		
	Meta myelocyte neutrophil	6 4 to 15 0	9 7	0 4 to 8 5	2 76	± 1 7257
	Staff or band neutrophil	14 6 to 38 5	5 3	12 4 to 60 0	33 91	± 9 1938
	Segmented neutrophil	6 5 to 25 6	16 8	0 0 to 23 6	5 35	± 5 0232
	Mature eosinophils	0 0 to 7 6	4 7	0 0 to 7 4	2 77	± 1 6539
	Basophils	0 0 to 1 0	0 2	0 0 to 1 2	0 08	± 0 090
B Non granular series	Lymphocytes	3 0 to 12 0	6 25	2 9 to 34 5	15 68	± 7 2623
	Large mononuclears	1 2 to 7 0	3 0	0 0 to 3 5	1 585	± 0 9386
	Plasma cells	0 0 to 1 6	0 6	0 0 to 1 5	0 31	

After Sabin School and not according to Israel s nomenclature which has been given in the text The term megalobla t is usc l here to denote the earliest differentiated percursor of both normal and abnormal red cells

counts forming 0 1 to 0 2 per cent of the nucleated cells In a properly drawn and well stained smear there are very few disintegrating cells and practically all the nucleated cells seen can be identified in a highly active marrow however there are some cells which show mitotic division and are difficult to identify properly

13
THE ARNETH AND SCHILLING COUNTS

Arneth (1904) divided the neutrophil polymorphonuclears into five main classes according to the number of segments in the nuclei which in their turn were classified into various groups according to the shape of the lobes Under the original classification the count was very complicated and had only limited application in general practice Cooke and Ponder (1927) modified and simplified the Arneth count taking into account only the five original basic divisions of the Arneth count without going into the intricate subdivisions of Arneth

Schilling devised a method in which the differential leucocyte count and a simple nuclear lobe count of the neutrophils were incorporated and considered in the form of a hæmogram

Apparatus required (*vide p 4 et seq*)

Smears —Smears for the Arneth and Schilling counts should be very thin and uniformly spread they should be dried quickly by being waved to and fro or held under a fan (*vide p 43*)

Staining —Combined staining with May Grunwald and Giemsa stains gives the most satisfactory result—the nuclei stain deeply and the connecting chromatin filaments are well shown while the granules also show characteristic colour and size If May Grunwald and Giemsa stains are not available proper staining with Wright s or Leishman s stains though not as satisfactory gives quite good results Iron hæmatoxylin or hæmatoxylin staining for these counts in preference to any of the Romanowsky stains is sometimes advocated while these stains bring out the nuclei and the chromatin filaments well the colour of the granules is not distinctive so that differentiation between neutrophils and eosinophils is very difficult

(A) Arneth count

The nucleus of the neutrophil polymorphonuclear cells is divided into lobes which may be separate or joined together by fine filaments of chromatin Cooke s criterion of separate lobulation is that either the lobes should be distinctly separate or should be joined together by a fine chromatin filament but not by broad bands of nuclear material Lobulation of the nucleus it is assumed gives usually an indication of the age of the cell—the fewer the lobes the younger is the cell The youngest cell in the normal peripheral circulation is one which has a nucleus shaped like the letter C while the cells with five or more distinct nuclei are the oldest

The principle of the method is to make a differential count of the neutrophil cell to group the cells according to the lobulation of the nuclei and to calculate the percentage of cells in each group

The cells of the various types can be described as follows —

Class I Neutrophil granulocytes in which the nucleus has only one definite lobe or two or more lobes joined by a definite band of chromatin and not by thin filaments

Class II When there are two separate lobes joined by a thin filament or when there are two completely separate lobes

Class III When there are three separate lobes connected by thin filaments or there are three completely separate lobes

Class IV When there are four separate lobes connected by thin filaments or there are four completely separate lobes

Class V When there are five or more separate lobes connected by thin filaments or there are five or more completely separate lobes

When in doubt about the number of lobes in cells with three or more lobes they must always be placed into the next lower class for example if there is any doubt whether a cell has three or four lobes it should always be placed in class III

The count is made by identifying and classifying one hundred consecutive neutrophil granulocytes from different parts of the slide and the result is usually reported in the following form —

Example 1 a normal count (European standard)

Class	I	II	III	IV	V	Total
Number	10	25	47	16	2	100

When there is an increase in the number of cells with one and two separate nuclear lobes i e of class I or class II at the expense of those with three or more lobes there is said to be a left shift in the Arneth count as in the example given below —

Example 2 a left shift count

Class	I	II	III	IV	V	Total
Number	41	44	14	1	0	100

Conversely when there is an increase in the 4— and multi lobed nuclei it is known as a right shift

Example 3 a right shift count

Class	I	II	III	IV	V	Total
Number	9	22	36	23	10	100

Various indices and methods of expressing more concisely the results of the Arneth count (Cooke s modification) have been proposed Probably the most suitable of these methods is the calculation of the weighted mean of the nuclear

lobes this is obtained by multiplying the number of cells in class I by 1 the number in class II by 2 the number in class III by 3 the number in class IV by 4 the number in class V by 5 and then adding together and dividing the sum total by the number of cells counted

The weighted means of the nuclear lobes in these three examples are calculated in the following way —

Example 1	Example 2	Example 3
1 × 10 = 10	1 × 41 = 41	1 × 9 = 9
2 × 25 = 50	2 × 44 = 88	2 × 22 = 44
3 × 47 = 141	3 × 14 = 42	3 × 36 = 108
4 × 16 = 64	4 × 1 = 4	4 × 23 = 9
5 × 2 = 10	5 × 0 = 0	5 × 10 = 50
100 275	100 175	100 303
Weighted mean =2·75	1·75	3·03

Interpretation —Immature granulocytes and cells of Arneth class I and some of class II but none of class III IV or V are found in the bone marrow If there be a sudden stimulus to the bone marrow to produce granulocytes a in an infection the leucocytosis is brought about by young cells appearing in the blood the number and the proportion of these depending upon the nature of the stimulu and the reacting power of the marrow of the individual and there is a shift to the left in the Arneth count But leucocytosis as a result of muscular exercise is only due to redistribution of cell is not accompanied by any increase of young leucocytes in the peripheral circulation and is therefore not associated with any left shift

Again in chronic infection or toxæmia there is an increased leucocyte destruction which may or may not be balanced by new leucocyte formation in the former case there will be leucocytosis or a normal count and in the latter leucopenia In either case there is an increase in cells of class I and II actual or relative which results in a shift to the left and a decrease in the weighted mean It will be apparent that this shift is quite independent of the total leucocyte or total granulocyte count and provides another indication of the degree of the toxæmia

In pernicious anæmia and certain other conditions of bone marrow dysfunction there is a shift to the right in the Arneth count and therefore an increase in the weighted mean in such conditions there are many multi lobulated cells and only very few young cells coming into the circulation

Normals

	I	II	III	IV	V	Weighted mean
Cooke and Ponder (1927)	10	25	47	16	2	2 75
Kennedy (1933) (Iraq)	13	30	43	10	4	2 62
Das Gupta (Calcutta)	37	44	16	3	0	1 85

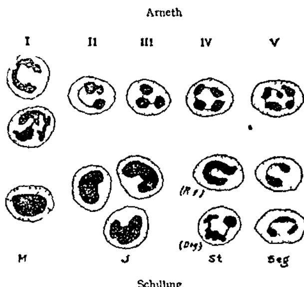
(B) SCHILLING COUNT

In this count the neutrophil granulocytes are divided into four groups and classified as follows —

1 Myelocytes (M)

2 Juveniles (J) these have a distinct indentation in the nucleus they correspond to our meta myelocytes (*vide* p 56 table XI)

3 Stab cells (St) these have a C – or horse shoe shaped nucleus but no true lobulation they correspond to class I of the Arneth count and to our staff cells



Schilling

Stab cells may be of the regenerative or degenerative type and are differentiated by the shape and staining reaction of the nucleus in the latter the nucleus is ribbon like and pycnotic with clumps of chromatin and usually irregular shaped and the cytoplasm may be vacuolated

Segmented (Seg) polymorphonuclears showing two or more distinct lobes

According to Schilling the following are the constituents of a normal hæmogram in a German population —

Total leucocytes	Percentages							
	B	E	M	J	St	Seg	L	Mon
5 000 to 8 000	0-1	1 2	0	0-1	3 5	57 67	21 35	4 8
			60 70					

B = basophil E = eosinophil
L = lymphocyte Mon = large mononuclear

Discussion —The Schilling hæmogram is much more than a differential granulocyte count as is the Arneth count and Schilling claims that it gives far more information The interpretation depends on a highly imaginative series of responses to infection and toxæmia that are supposed to and possibly do occur in the hæmopoietic system in which there are three phases neutrophilic struggle monocytic defence and lymphocytic cure but it does not seem to take into account the nature of the infection and the consequent variations in the response Further there are so many directions in which changes may take place that the hæmogram seems peculiarly susceptible to facile interpretation to suit the conscious or unconscious wishes of the interpreter

We do not propose to give a full discussion on the interpretation of the hæmogram as it has only an indirect bearing on the subject of anæmia but as far as the neutrophil granulocyte elements are concerned Schilling s contention is that infection may cause two types of reaction the regenerative reaction and degenerative reaction in the regenerative reaction new leucocytes are formed in the bone marrow and this is reflected in the peripheral circulation by a rise in the total white cell count an increase in the juveniles and regenerative forms of stab cells while in the degenerative reaction which is found in severe toxæmia on account of the degenerative influence of the toxin on the marrow the formation of new cells is retarded this may be shown by a leucopenia In the differential count of such a case there are few juvenile cells and the stab cells that reach the circulation fail to segment at the usual rate and may degenerate with the result that there is a preponderance of stab forms including many degenerative form in the blood The reaction however may not be purely regenerative or degenerative in character but may be a mixture of the two and the hæmogram may be intermediate with slight leucopenia and a greater preponderance of the younger cells than in a purely degenerative process

Normals

	I	II	III	IV	V	Weighted mean
Cooke and Ponder (1927)	10	25	47	16	2	2 75
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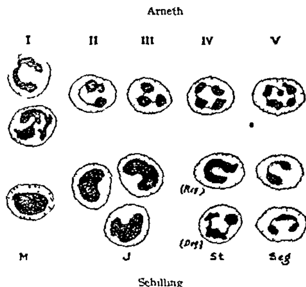
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Stab cells may be of the regenerative or degenerative type and are differentiated by the shape and staining reaction of the nucleus in the latter the nucleus is ribbon like and pycnotic with clump of chromatin and usually irregular shaped and the cytoplasm may be vacuolated

WESTERGREN S METHOD

Apparatus required

(i) Sedimentation tubes
Westergren tube This is about 300 mm long and is graduated from 0 to 200 mm in 1 mm interv l it has a uniform bore of about 3 mm and is open at both ends
( ) Special West rgren rack or an improvised rack to keep the tubes in a vertical position and to prevent the blood escaping
( ) Anti-coagulant—3 8 per cent sodium citrate solution
(iv) Syringe etc for collection of blood
(v) Stop watch

Technique —In this method 3 8 per cent solution of sodium citrate is used as anti coagulant in the proportion of one part of citrate solution to four parts of the blood

Put exactly 2 c cm of blood into a flask to which 0 5 c cm of 3 8 per cent solution of sodium citrate has already been placed Mix immediately by rotating the flask and subsequently if possible in a shaker then draw up the mixture into a Westergren s standard tube to the zero mark which is exactly 200 mm from the tip The tube is now set upright in a stand in which a spring clip holds the points of the tube firmly against a rubber cork at the bottom end The tube is now l ft to stand and the upper level of the red cell column which is generally sharp is read at the end of one and again after two hours* The length of the column of plasma from the zero mark to the top of the red cell column is the amount of edimentation and this is read off in millimetres this is reported as the sedimenta tion rate in millimetres for one hour and for two hours

Norm al limits (afte one hour)—

	Men	Women
Textbooks	3 to 5 mm	4 to 7 mm
Our o vn experience in Indians n Calcutta	3 to 15 mm	5 to 40 mm

The high figures that we obtained in apparently normal subjects are partly explained by the higher temperatures that prevailed compared with those of the places where the tests on which other normal figures are ba ed were carried out

WINTROBE S METHOD

Apparatus requ red

( ) Wintrobe s tubes—a fl t bottomed glass tube 115 mm long graduated from 0 to 100 in 1 mm intervals it has a uniform bore of 2 5 mm
(i) Anti-coagulant—sodium and potass um oxalate mixture (*vide* p 32)
(i) Electric centrifuge

Together with a syringe etc and stop watch as in Westergren s method

Technique —Wintrobe used his cell volume tube for the determination of the sedimentation rate

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* Only one hour readings are usually recorded

(It is surprising to the writers that no provision is made in the classical Schilling hæmogram for the separate counting of the regenerative and degenerative forms of stab cell when obviously the presence or absence of the latter is an important factor in the interpretation of the hæmogram)

Conclusion —The interpretation of the Arneth count is simpler though the *implications are more limited* but it presents a minor practical objection in that more skilful staining is required to ensure accurate counting of the separate lobes

The value of these counts is mainly *in establishing the presence of infective or* toxæmic factors which may be important contributory causes of the anæmia and/or may prevent response to appropriate hæmatinics The presence of multilobular cells shown only *in the Arneth count is important in the diagnosis of* pernicious anæmia

14

THE ERYTHROCYTE SEDIMENTATION RATE

The determination of the erythrocyte sedimentation rate (ESR) is not a test that gives much direct information regarding the nature of the anæmia but it is of value in differentiating functional disorders such as pernicious anæmia in which the true ESR is within normal limits from anæmia due to such causes as sepsis and malignant disease in which the ESR is markedly increased

Further as from the single specimen taken in the normal course of a routine blood examination the ESR can be estimated without additional labour it should always be included

The test suffers from the multiplicity of the methods by which it is performed and by the variety of the forms in which the results are expressed The most commonly used technique is that of Westergren (1926) but as it is important that the correction for anæmia should be made and as no correction chart has been worked out for this technique this method has its limitation

Wintrobe s method (1933) is really a modification of this method it has the advantage that the cell volume can be estimated on the same sample of blood and also that a correction for anæmia can be made

Principle of the test —A known volume of blood is intimately mixed with a definite amount of anti coagulant and the mixture is drawn into a tube of known calibre up to a certain point the tube is then allowed to stand in an upright position at room temperature and the level of the red blood cells *in the tube is noted* at the end of one hour and again after two hours The fall in the level of the red cells is expressed in millimetres

To make the correct allowance for this fact Hynes and Whitby (1938) worked out a chart (fig 20) showing the relationship of cell volume and sedimentation rate in normal blood and in blood showing various degrees of anæmia

Normal limits (after one hour)—

	Men	Women
Textbooks	0 to 9 mm	0 to 0 mm
Our own experience in Indians in Calcutta	2 to 20 mm	2 to 30 mm

Precautions

(*i*) All apparatus must be scrupulously clean and dry

(*ii*) The dilution with sodium citrate solution in Westergren s method should be absolutely correct

(*iii*) The specimen of blood should be collected during the fasting state and examined within one hour of collection

(*iv*) In women it is advisable to avoid the menstrual period and the few days before and after it

(*v*) The specimen should be rejected if there is a slight trace of coagulation or any marked hæmolysis

(*vi*) The tubes should be set up in an absolutely vertical position in a quiet place well away from any apparatus that vibrates The temperature of the room should be between 72 and 80 F whenever possible

(*vii*) In recording the results correction for anæmia must always be applied

Discussion —There are a number of factors involved in the phenomenon of erythrocyte sedimentation but the most important is the size of the rouleaux formation and this is mainly determined by the composition of the plasma and to a certain extent by changes in the shape and size of the red cells Increase in plasma fibrinogen will cause an increase in the size of the rouleaux formation and therefore an increase in the sedimentation rate It is probably on this fact that the value of this test depends

Erythrocyte sedimentation rate is also greatly influenced by changes in the concentration of the red cells in the plasma that is any associated anæmia This may overshadow other changes but being a measurable factor must be allowed for in estimating the true sedimentation rate

The sedimentation rate is increased in so many conditions that it is very dangerous to attach any specific diagnostic significance to it This must be quite obvious from the wide range of the ESR rates that has been recorded in apparently healthy individuals However the ESR is a test of great value in estimating the progress in chronic infections such as tuberculosis and leprosy A standard technique should be adopted and in reporting the results the method employed must be stated or the information loses much of its value

The blood is collected in an oxalated flask and is thoroughly mixed the cell volume tube is filled with the oxalated blood by means of a capillary pipette up to the zero mark which is exactly 100 mm from the bottom The tube is now allowed to stand upright in a vertical position on a special stand or on a tray of plasticine and the reading is taken at the end of one and again after two hours in the same way as in Westergren s method the cell volume tube is then put into a centrifuge and centrifuged at 3 000 revolutions per minute for half an hour to obtain the packed cell volume percentage thus both procedures are done in the same tube with the same blood sample

Correction for anæmia —One important factor in the rate of sedimentation of bodies in fluid is the concentration of these bodies The sedimentation standards for red cells in plasma are based on there being a normal number of red cells in a given volume of blood and in anæmia when these are reduced the sedimentation rate is increased above the normal irrespective of the other changes in the blood

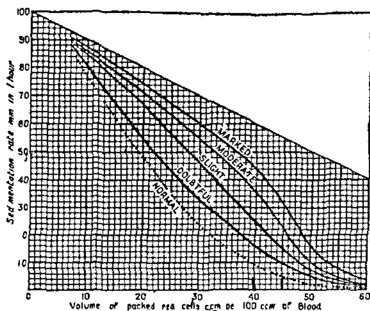


FIG 20 —Chart for correction of sedimentation rate for anæmia by means of the corpuscular volume

To correct for anæmia find the junction of the lines of the observed sedimentation rat and the observed corpuscular volume in the chart this point will fall in on of the five zones (normal doubtful etc) which indicate the approximate degree of increase in the rate If a compensated figure is required follow the appropriate curve dow 1 to the point where it cuts the 45 c cm vertical lines for men and the 40 c cm line for women

Example —Observed rate = 50 mm observed corpuscular volume = 30 per cent point of junction lies in area of slight increase compensated rate for men = 18 and for women = 28

Fractional gastric analysis gives valuable information as to the motility and secretory functions of the stomach This method is followed by us in the investigation of all cases of anæmia in the hospital and in many of the cases attending the anæmia out-door clinic

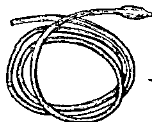


FIG 21

In the course of the fractional gastric analysis an injection of 0 5 mg of histamine is given one hour after the test meal to the patients who fail to show any free acid in any of the previous specimens

Apparatus required

(i) Rehfuss or Ryle s tube or any suitable modification (*vide* figure 21) These are tubes made of good rubber They are about 3 inches long with and internal diameter of about ⅛th inch One end of the tube is open while the other the tip ends in an olive shaped bulbous protuberance filled with metal to weight it and keep it rigid There are holes in the rubber at or near the tip for the passage of the gastric juice through the tube The distance up to which the tube has to be passed is shown by a mark near the open end about 20 to 22 inches from the tip

Before use the tube is sterilized in boiling water for a minute or two and is kept coiled up in a sterilized petri dish until required

(ii) Record syringes 10 c cm and 1 c cm
(iii) Twenty five c cm burette graduated in 1/10th of a c cm with a stand
(iv) Twelve labelled test tubes in a rack *plus* a few extra tubes
(v) Capillary pipettes with teats
(vi) Small glass funnel
(vii) Porcelain dishes—3
(viii) Glass rods—3
(ix) Centrifuge machine
(x) Slides
(xi) Microscope

Chemicals required

(i) One per cent solution of cocaine or a suitable substitute
(ii) Seven per cent alcohol 100 c cm
(iii) N/10 sodium hydroxide
(iv) Dimethyl amido azobenzene 0 5 per cent in 95 per cent alcohol (Töpfer s reagent)
(v) Phenolphthalein 1 per cent in alcohol
(vi) Histamine—0 5 mg in 0 5 c cm sterilized distilled water
(vii) Adrenaline chloride solution 1 in 1 000 for injection
(viii) Sulphur powder
(ix) Benzidine
(x) Glacial acetic acid
(xi) Hydrogen peroxide
(xii) Pepsin
(xiii) Mett s albumin tubes (*v i*)
(xiv) Dilute hydrochloric acid
(xv) Fresh milk
(xvi) Lugol s iodine solution

ROUTINE PROCEDURE

The following is our usual procedure in carrying out a fractional gastric analysis —

A Preparation of the patient

On the previous night the patient has his usual meal at 8 p m and at 10 p m he is given four charcoal tablets with a glass of milk The next morning he is not

15
GASTRIC ANALYSIS

Introduction —The gastric juice in the normal individual contains hydrochloric acid free and in the combined state the enzymes pepsin and rennin and the intrinsic factor of Castle Examination of the aspirated gastric juice primarily for acidity but also for the presence of the enzymes and for other normal and abnormal characters is known as gastric analysis

We have included gastric analysis in this series on hæmatological technique because normal hæmopoiesis is to a large extent dependent on normal gastric function and consequently the knowledge that we obtain from this test is important in both the diagnosis and the treatment of the anæmias

Achlorhydria or hypochlorhydria is associated with deficient digestion and absorption of a number of food substances In some cases of microcytic anæmia achlorhydria is considered to be the main ætiological factor iron is more easily absorbed from an acid than from an alkaline medium Free acid is necessary also for ensuring proper peptic digestion (*v i*) Further in the absence of hydrochloric acid fermenting organisms flourish causing flatulence meteorism and diarrhœa This mucosal dysfunction leads to a macrocytic anæmia the anæmia of sprue is often of this type and due to this cause but neither in sprue nor in nutritional macrocytic anæmia is achlorhydria constant nor is there any evidence that the *intrinsic factor is also absent Finally in pernicious anæmia there is complete* and constant achylia * which is associated with the absence of the intrinsic factor though this latter deficiency cannot be demonstrated directly in the laboratory

*In practically all cases of anæmia associated with achlorhydria or hypochlo*rhydria the giving of dilute hydrochloric acid alone or with pepsin will be beneficial and may in fact constitute an important part of the treatment

Methods Gastric analysis can be done by *either of the following methods* —

(i) Single examination This is an old method and is obsolete now In this method after complete evacuation of the fasting juice an *Ewald meal consisting of bread and water* is *given to the patient* and the stomach content withdrawn again after one hour

(ii) Fractional analysis of Rehfuss In this method after complete evacuation of the fasting juice a suitable test meal is given to the patient small samples about 10 c cm of the gastric fluid are drawn off every 15 minutes up to 2½ to 3 hours

*The following terms are in common use for the different stages of gastric deficiency —

Achlorhydria (*or false* achlorhydria)	= absence of free hydrochloric acid during an ordinary test meal in which histamine was not given
Complete achlorhydria	= absence of free acid even after histamine injection
Achylia	= absence of free acid and the gastric enzymes even after histamine

After complete evacuation of the stomach contents the test meal is given to the patient with the tube *in situ*

D Test meals

An ideal test meal is obviously one that bears a close similarity to the ordinary diet of the patient but for many reasons it is not possible to give such a test meal Various test meals have been advocated but here we shall describe only two with which we have had personal experience

(i) Gruel test meal In making this we have always used Quaker oats but any form of prepared oats can be used and in this country some workers prefer to use a rice gruel Take a tablespoonful of Quaker oats in two pints of water add a pinch of salt boil down to a pint and strain through fine muslin This meal does not contain any lactic acid and is thus almost an ideal test meal but with the tube *in situ* it becomes very difficult and sometimes impossible to swallow such a large quantity of thick gruel

(ii) Alcohol test meal One hundred c cm of 7 per cent alcohol is used for this meal The measured quantity of alcohol is placed in a beaker from which it is drawn up into a syringe and introduced into the stomach through the tube the process is repeated until the whole amount has been introduced

This meal is very easy to administer while the fluid that is subsequently withdrawn is almost clear this allows of easy titration for acidimetry

S Withdrawal of post prandial specimens

Note the time when the test meal is given aspirate with a syringe about 10 c cm of gastric contents every 15 minutes up to 2½ or 3 hours The specimens are kept in labelled test tubes until the time of examination

Histamine —As the fasting and post prandial juices are withdrawn they are examined for the presence of free hydrochloric acid by the bedside

Take one c cm of the gastric juice in a small test tube add a small drop of Topfer's reagent Note the colour—red or orange colour indicates the presence of free hydrochloric acid and further bedside examination of subsequent specimens is not necessary

If free hydrochloric acid is not present in the fasting juice and in the first four post prandial specimens an injection of 0 5 mg of histamine is given and the procedure of withdrawing samples is continued as before A little flushing of the face is seen after injection of histamine and occasionally the patient may complain of palpitation which generally pass off quickly In the event of the patient becoming distressed by these symptoms an injection of 0 5 c cm adrenalin chloride should be given this will give instantaneous relief

allowed any food or drink before the test is finished Indian patients must be warned not to chew any *pan* as it may impart a red colour which might be mistaken for blood to the gastric juice

Before introducing the tube in very sensitive patients the nasal mucous membrane and the posterior part of the pharynx may be sprayed or swabbed with 1 per cent solution of cocaine or with a suitable substitute

B Introducing the tube

(i) *Through the mouth* With a patient in a comfortable position sitting if possible, but the test is best done with the patient lying down the tip of the tube is placed on the posterior portion of the dorsum of the tongue and allowed to drop slowly backwards and downward When the tip strike the posterior pharynx the patient is instructed to make swallowing movements and the tip will then pass into the œsophagus The patient should be told to continue to swallow slowly when the tube will gradually descend until the end has reached the fundus of the stomach the mark on the tube gives an approximate indication of when this point is reached

(ii) *Through the nose* With a little practice the introduction of the tube through the nose is much easier than through the mouth and can be carried out even in the most sensitive patient The tip of the tube is introduced into one of the nasal orifices and gently pushed through the nose until it reaches the posterior wall of the pharynx The patient is now told to make swallowing movements while the tube is pushed gently down until it reaches the fundus of the stomach

Some obstruction may be felt in passing the tube through the nose this is easily overcome by a little manipulation but if the resistance is great the tube must be taken out and introduced through the other na al orifice as not infrequently the septum is deviated to one side

C Drawing out the contents of the fasting stomach

When the tube has been introduced up to the required distance introduce the nozzle of a 10 c cm syringe into the tube with the piston drawn out push down the piston so that the air in the syringe is forced through the tube to dislodge any mucus or food debris that may be blocking it at its distal end then aspirate the contents of the fasting stomach If there is any difficulty in getting the juice vary the position of the tube in the stomach by drawing it out or pushing it in and/or by forcing more air through the tube

Rarely difficulty in obtaining juice may be due to contraction of the gastric muscles which may be difficult to overcome An attempt should be made to draw out all the fluid of the fasting stomach—by altering the position of the tube in the stomach by putting the patient in different postures and by applying a little pressure to the stomach from outside The contents of the stomach are placed in the test tubes previously labelled

(a) Estimation of free hydrochloric acid

Titrate with N/10 sodium hydroxide until the red or orange colour is discharged; this is done as follows:—

Fill a graduated 25 c.cm. burette with N/10 NaOH up to the zero mark. With one hand regulate the flow of NaOH and allow it to fall drop by drop from the burette into the porcelain dish; stir all the time with a clean glass rod with the other hand. The end point will be shown by a brownish and not a yellow colour. Take the reading of the burette; the difference between the two readings (the first should be zero) gives the amount of NaOH that was required to neutralize 5 or 10 c.cm. of gastric juice.

In clinical work the result is always expressed as the number of cubic centimetres of N/10 NaOH which would be required to neutralize 100 c.cm. of the gastric juice, each cubic centimetre representing *one degree* of acidity. So the number of degrees of acidity is calculated by multiplying the number of cubic centimetres of N/10 NaOH by 10 if 10 cubic centimetres of gastric juice was used, or by 20 if 5 c.cm. of gastric juice was used.

The result may be expressed in grammes of hydrochloric acid by multiplying the number of degrees of acidity by 0.00365 (an easy way to remember this is that 365 is the number of days in the year, the last figure—5—indicating the number of decimal places).

Example.—If 10 c.cm. of gastric juice was taken and if the end point was reached when 2.3 c.cm. of N/10 NaOH had been added, the degree of acidity is is 2.3 × 10 = 23.
And in terms of hydrochloric acid 23 × 0.00365 = 0.08395 g. of hydrochloric acid.

(b) Estimation of total acid

After the estimation of free hydrochloric acid, or if there is no free HCl at all, 1 or 2 drops of phenolphthalein (1 per cent. solution in alcohol) is added as an indicator and the titration with N/10 NaOH is continued until a permanent faint red colour develops.

The third reading *minus* the first reading (zero in this case) multiplied by 10 if 10 c.cm. was taken, or 20 if 5 c.cm. was taken, gives the degree of total acid.

The total acidity is made up of free HCl together with the HCl which is combined with protein and mucus, *plus* the organic acids, such as lactic and butyric, which result from fermentation.

The estimation of total acidity is of little practical importance. It is usually about 10 degrees higher than the free HCl, except in cases of marked lactic or butyric acid fermentation, when it may be very much higher than the free HCl. In these cases the typical rancid butter odour enables one to detect fermentation.

(c) Lactic acid

Test for lactic acid must be applied in all cases where there is no free HCl in the fasting juice.

Examination of Gastric Contents

A Macroscopic examinations

(a) *In the fasting juice*

(i) Amount measure and note the amount Normally 20 to 25 c cm are found Marked increase over 50 c cm suggests hypo motility obstruction or hypersecretion

(ii) Odour normally it has no striking odour An offensive odour suggest cancer and a sour odour fermentation

(iii) Remnants of food or charcoal normally no food remnants or charcoal are found after 10 hours interval The presence of food remnants or charcoal particles suggests hypo motility pyloric obstruction or ptosis

(b) *In the fasting juice and in the post prandial specimens*

(iv) Mucus in large quantity in the fasting juice and in many of the later post prandial specimens indicates catarrhal gastritis

(v) Bile traces of recently regurgitated lemon yellow bile may be seen in a few specimens and are usually due to retching caused by the introduction of the tube A large quantity of turbid green bile in the fasting and in any of the early post prandial samples is almost always pathological

(vi) Blood macroscopic examination for blood gives more valuable information than the chemical examination Flecks of fresh blood are usually the result of trauma in passing the tube while large quantities of fresh blood in any specimen would indicate varices erosions or even malignant ulceration of the œsophagus Blood from a gastric ulcer or carcinoma of the stomach is changed to brown acid hæmatin by the acid in the stomach juices it is found most frequently in the fasting juice but it may be found also in any post prandial specimen

B Chemical examinations

(i) *Acidimetry*

Place 5 or 10 c cm of clear gastric contents in a shallow porcelain dish If there is an excess of mucus in any specimen filter the gastric juice through a plug of cotton wool to remove the mucus this will then allow of easy titration

Add a drop of Topfer s reagent to the gastric juice in the porcelain dish the presence of free hydrochloric acid will be indicated by the red or orange colour of the juice

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Interpretation —Digestion in tube 2 indicates the presence of both pepsin and free hydrochloric acid in the gastric juice

If digestion fail in tube 2 but occurs in tube 3 pepsinogen is present but it required the free HCl to be added to the tube to convert it into pepsin

If there is no digestion even in tube 3 then both pepsin and pepsinogen are absent

(ii) *Detection of rennin* —The enzyme rennin coagulates the protein of milk Fresh milk i used as the reagent

Neutralize 5 c cm of clear gastric fluid with very dilute sodium hydroxide Add an equal amount of fresh milk and place in a water bath at 40 C for 15 minutes

Interpretation —Coagulation of the milk in 10 to 15 minutes denotes the presence of a normal amount of rennin delayed coagulation denotes a decreased amount

D Microscopic examination of the gastric residue

This examination ha very limited application and need only be done if carcinoma is suspected The fasting contents when no free HCl is present are mo t suitable for the test

Centrifugalize the fasting contents take a small loopful from the bottom and smear it on a glass slide Fix by heat and stain by Lugol s iodine and examine with the oil immer ion lens for Boas Oppler bacilli

Boas Oppler bacilli are large (5 to 10 μ long) non motile and usually arranged in clumps or end to end in zig zag chains They stain yellow to brown with iodine solution which distingui hes them from *Leptotrichia buccalis* which is not infrequently swallowed and hence found in stomach fluid (Todd and Sanford 1939)

Method of recording results

The results are best recorded on a pecial chart which may be printed or cyclostyled

Figure 2 shows the form of chart that we use except that in this figure a shaded area has been added to show the range within which 80 per cent of gastric analyses in normal individuals fall

Both free acid and total acid are recorded in the chart preferably in different colours The presence of mucus bile and blood is indicated by a *plus* or a double *plus* sign in the appropriate place

Add 2 drops of 5 per cent aqueous solution of ferric chloride to half an inch of clear gastric fluid in one tube and to half an inch of water in another tube of the same size which will serve as a control To each add 6 drops of saturated solution of mercuric chloride If lactic acid is present in the gastric contents a deep yellow colour will be produced in that tube but not in the other

(ii) *Bile*

The presence of bile will be denoted by the colour in the gastric fluid If in doubt Hay s sulphur test can be done by sprinkling sulphur dust on the surface of a specimen of clear juice If bile salts are present the sulphur will fall to the bottom

(iii) *Blood*

Dissolve a little benzidine in 2 c cm of glacial acetic acid warm if necessary add 2 c cm of 3 per cent hydrogen peroxide Finally add 1 c cm of the gastric juice and mix well by shaking A blue or bluish green colour indicates the presence of blood

The test is very sensitive and will detect blood in dilution of 1 3 000 000

C *Examination for enzymes*

(i) *Detection of pepsin* —Normally pepsinogen is secreted by the stomach and is transformed into pepsin by the free HCl in the stomach Its presence is detected by the digestion of egg albumin

Method —Take three small test tubes

In tube 1 put 1 g of pepsin 2 c cm of the HCl solution and a one inch length of capillary tube of albumin *

In tube 2 put 2 c cm of clear gastric fluid and a capillary tube of albumin

In tube 3 put 2 c cm of clear gastric fluid 2 c cm of the HCl solution and a capillary tube of albumin

Place all the three test tubes in a warm incubator overnight examine them for digestion of the albumin next morning

Tube 1 in which digestion should be complete serves as a control

---

* Mett s capillary tubes of albumin are prepared in the following way —

Mix the white of 3 or 4 egg beating them gently

Make some capillary *tubes of uniform bore Fill the tub with the egg a* lbumin and cut them into pieces of about 10 to 1 inches long Seal the ends with ealing wax Boil a larg quantity of water in a big vessel Just as the water begin to boil put the capillary tube containing albumin into the boiling water Remove the vessel from the source of the heat After 5 minutes take out the capillary tubes—the egg albumin will be lightly coagulated Store the tubes in a refrigerator

Before use cut *the egg albumin* tubes into length of one inch taking care that the ends are clean cut and circular

These criteria are based on our personal experience in India (Napier and Das Gupta 1935, Napier Chaudhuri and Rai Chaudhuri 1938) but they do not differ materially from those adopted by workers in other countries. About 80 per cent of normal individuals fall within the isochlorhydria range; within this range women will usually be lower than men (figure 23). There is no evidence that the gastric acidity of Indians in India is lower than that of Europeans in their own countries. The data available from our own experience suggest that in South Indians and Bengalis (figure 24) the acid range is a little higher than the textbook figures which are based on European and North American subjects.

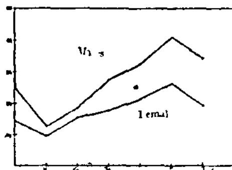


Fig 23 —A sample curves in males and females (gruel test meal)

From 2 to 4 per cent of apparently normal Indians are completely achlorhydric even with histamine; this is in keeping with experience in other countries.

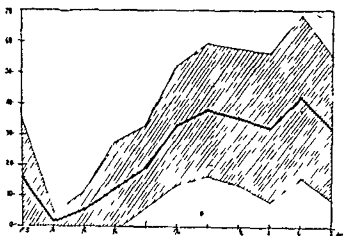


Fig 24 —Normal Indians in Calcutta (gruel test meal)

With the alcohol test meal the acid curve on the whole tends to be higher; this is particularly noticeable in the first post prandial sample in which the initial drop in acidity, due mainly to the neutralization of the acid by the meal, may be absent. Another difference is that the maximum acidity is usually reached earlier when alcohol is given as a test meal.

Normal standards

Acid curves will vary not only in height but in shape and one cannot judge them solely on one feature, e.g. on the highest point reached during the test.

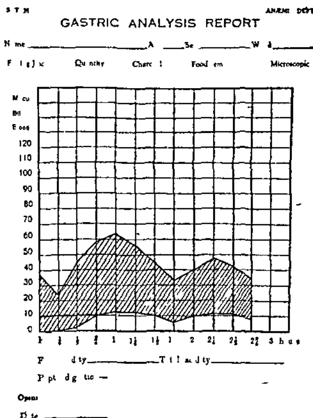


FIG. 22

Nevertheless, if a curve is to be classified on a single feature this is probably the best one, as it usually reflects fairly accurately the rest of the curve. We have adopted the following criteria for classifying acid curves according to the highest free acid reading:—

	c.cm. of N/10 NaOH required to neutralise 10 c.cm. gastric juice
Achlorhydria	0
Hypochlorhydria	< 10
Isochlorhydria low	10 to < 25
medium	25 to 4
high	< 45 to 65
Hyperchlorhydria	< 65

the numbers of the two tubes that show the beginning of hæmolysis and complete hæmolysis are noted and from these the strength of the corresponding saline solutions are calculated

Apparatus and reagents required

(i) Accurate solutions of sodium chloride 0·5 per cent and 1·0 per cent standardized against silver nitrate solution
(ii) Freshly boiled distilled water (pH 7·0)
(iii) Small test tubes
(iv) Metal rack for holding the tubes
(v) 5 or 10 c cm syringe with needle of fairly wide bore
(vi) Capillary pipette with teat

A convenient form of rack is that used for the Wassermann reaction The holes are in two rows with single tubes at either end of the rack The holes are numbered 12 to 24 and those at each end S and W (figure 25)

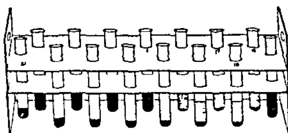


FIG 25

With a syringe fitted with a needle of fairly large bore or with a capillary pipette put into each tube the number of drops of 0·5 per cent sodium chloride solution indicated by the number on the rack opposite that tube and 25 drops in the tube marked S

Wash out the syringe or the pipette thoroughly with distilled water With the same needle or pipette add a sufficient number of drops of distilled water to bring the total number of drops of fluid in each tube up to 25 and put 25 drops of distilled water in the tube at the end of the rack marked W

Examples—

Numbers on racks opposite test tubes	W	12	13	14	15	16	17		24	S
Number of drops of 0·5 per cent NaCl to be put into each test tube	0	12	13	14	15	16	17		24	25
Number of drops of distilled water to be added to each test tube	25	13	12	11	10	9	8		1	0
Concentration of NaCl (per cent)		0·24	0·26	0·28	0·30	0·32	0·34		0·48	0·50

Other indications given by gastric analysis

In chronic gastritis in the early stages there is hyperacidity and hypersecretion this latter is shown by the large quantity of fasting juice but in the later stages there is gastric atrophy with accompanying hypoacidity or achlorhydria

In gastric ulcer there is usually increased acidity and blood may be present

In gastric carcinoma the findings are sometimes very characteristic but in the early stages there may be no indications so that it is dangerous to exclude carcinoma on the strength of a normal gastric analysis There is achlorhydria excess of lactic acid and usually blood and the Boas Oppler bacillus is often present If the cancer is at the pylorus there may be delayed emptying and food or charcoal will be found in the fasting juice which will usually be very sour and offensive

In gastric neuroses there is usually hypersecretion and in dilatation of the stomach food retention and fermentation

16
THE RED CELL FRAGILITY TEST

Introduction —When normal red cells are suspended in 0 85 per cent saline they remain unaltered but they become crenated when the concentration of the saline is raised above 0 9 per cent whereas they are hæmolysed or laked when the concentration is lowered below 0 45 per cent

The study of the reaction of the red cells in hypotonic saline solution is known as the fragility test of the red cells

The fragility of the red cells is influenced by the CO content of the blood arterial blood is less fragile than venous blood while the fragility of the capillary blood is intermediate

Further the red cell is to some extent protected by its own plasma from saline hæmolysis which is more marked in case of anæmic patients (Creed 1939)

The test is conveniently done with fresh oxalated venous blood collected for routine examination of the blood (p 1) but for reasons given above the test should be done with washed red blood cells which have been thoroughly oxygenated by rotating the blood around in a wide mouthed bottle for over 5 minutes

The fragility of the red cells is expressed in terms of percentages of sodium chloride solution namely the points at which hæmolysis commences and at which it is complete

The test can be done either by a qualitative or quantitative method

Qualitative method

To each of a series of numbered test tubes containing saline solution of different graded concentrations one drop or two drops of blood are added the contents of the tubes are mixed well and allowed to stand for about 2 hours after which

Apparatus required

As in red-cell count (p 10)

Also accurate solution of sodium chloride (standardized against silver nitrate solution) from 0 3 to 0 7 per cent with intervals of 0 05 per cent

Technique—Fill red cell pipettes as in red-cell count using different strengths of salt solution ranging from 0 3 to 0 7 per cent with interval of 0 05 per cent Allow the pipette to stand on an even surface for about 15 minutes shake it well and then count the red cells in the usual way (*see* enumeration of red cell p 13 et seq)

Repeat this count with each solution and include a control with the usual diluent

From the above counts calculate the number of cells that become hæmolysed in different strengths of the salt solution and plot a curve The normal limit of fragility of the red blood cells as determined by the quantitative method of Whitby and Hynes (1935) is shown in figure 26

Discussion

The fragility test is based on the assumption that there is some parallelism between the fragility of the erythrocytes *in vitro* and their susceptibility to haemolysis *in vivo* Though it is very unlikely that the mechanisms of these two processes neither of which is fully understood are identical there is the evidence that in certain conditions e g acholuric jaundice where the red cells are particularly liable to hæmolysis *in vivo* they are hæmolysed by hypotonic saline solutions that would not affect normal erythrocytes

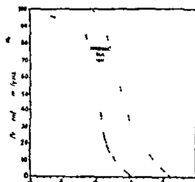


FIG 26—Fragility of red corpuscle as determined by quantitative method (Whitby and Hynes 1935)

In the physiological process of red cell demolition there is some evidence that the red cell normally a biconcave disc becomes more spherical in shape before it disintegrates and in many hæmolytic anæmias it has been shown that the red cells are more spherical than the normal red cells These spherical cells appear to be more susceptible to hæmolysis by hypotonic salt solutions but it is certain that this is not the only change that brings about this increased fragility

In all cases of obscure anæmia it is certainly worth carrying out the fragility test by the quantitative method if this is feasible not only in the interests of accurate diagnosis but in order to add to our knowledge of the ætiology of the many types of anæmia about which our knowledge is incomplete

Shake each tube thoroughly to get a uniform mixture To each tube add one drop or two drops if the patient is very anæmic of oxygenated blood (v s)

Shake all the tubes again Place the rack with the tubes on a flat even surface and allow it to stand for about 2 hours at room temperature If there is any special need for hurry centrifuge all the tubes for about 10 minutes and take the readings at once If possible put up a parallel test with normal blood as a check to the accuracy of the sodium chloride solution

Reading the results —Hold the rack with the tubes level with your eyes against daylight Note the number of the tube in which hæmolysis is first shown and also the number of the tube in which it is quite complete -Examine the tube commencing from tube No 25 and proceed towards the other end the first tube showing a tinge of reddish colour in the supernatant fluid indicates the beginning of hæmolysis and the first tube in which all the corpuscles are laked and no corpuscular residue is visible even after centrifugaligation indicates the point of complete hæmolysis

The result is expressed as a percentage of sodium chloride solution and is calculated by multiplying the figures on the tubes by 0 02

Example —The first tube in which the supernatant fluid shows any pink colour is numbered 20
The first tube in which there is no corpuscular residue is numbered 15
Therefore hæmolysis begins at 20 × 0 02 = 0 40 per cent saline
hæmolysis is complete at 15 × 0 02 = 0 30 per cent saline

When the test has to be carried out with very fragile cells e g in acholuric jaundice concentration of sodium chloride of over 0 5 per cent may be required in such a case use 1 per cent solution instead of 0 5 per cent and proceed with the test in the same way as before Take the readings and multiply the numbers on the tubes by 0 04 to get the percentage of the saline

Normally partial hæmolysis begins at about 0 44 per cent and is complete at about 0 30 per cent saline

Quantitative method

The qualitative method would be quite accurate if all the cells in any particular sample were of the same fragility but the individual cells vary in their fragility just as they vary in their size It is therefore important to know what percentage of cells have been hæmolysed by each solution This quantitative method is more laborious but is worth doing in special cases

Principle —A number of accurate red cell counts are done in the usual way except that instead of the standard diluting fluid different strengths of salt solutions* are used from the results an accurate quantitative graph of the fragility is plotted

* Simmel (1923) used mixtures of salts identical with those in the blood but Whitby and Hynes (1935) obtained identical results by using sodium chloride solutions we recommend the latter method on account of its simplicity

Known causes of the hæmorrhagic state are —

(i) Deficiency of platelets (and con equent deficiency of thrombokinase) as in thrombocytopenic purpura

(ii) A defect of the platelets so that they do not disintegrate at the usual rate as in hæmophilia

(iii) A deficiency of prothrombin in the blood as in vitamin K deficiency

(iv) Fibrinogen deficiency as in prolonged chronic hæmorrhage

(v) A defect in the capillary wall as in vitamin-C deficiency

In (i) there is a decrease in the platelet count a normal coagulation but a prolonged bleeding time and poor clot retraction the prothrombin is normal

In (ii) the bleeding time is normal and the coagulation time is prolonged

In (iii) the bleeding time is normal and coagulation time prolonged prothrombin is decreased but the clot retracts normally

In (iv) the bleeding time is normal and the coagulation time prolonged and *the clot retraction is defective but prothrombin is normal*

In (v) the bleeding time is prolonged and the capillary resistance i decreased but the coagulation time prothrombin and clot retraction are normal

A COAGULATION TIME

Various methods have been described for the determination of the coagulation time but it is essential to adopt one method and to follow the technique as uniformly as possible for the normal coagulation time varies very considerably with the technique with the temperature and with the diameter of the tube used * it is also influenced by admixture of the blood with tissue juice or any other foreign substances It i advisable first to determine the coagulation time of a few normal persons in each laboratory all subsequent examinations must be done exactly in the same way and the results interpreted in relation to the normal finding

Two methods are described as we have had considerable experience with each —

I CAPILLARY TUBE (WRIGHT S) METHOD

Apparatus and reagents required

(i) Fine capillary tube with diameters from 0 8 to 1 2 mm and six inches long
(ii) Pricking needle or blood gun
(iii) Water bath or warm water in a beaker at 37 C with a thermometer *
(iv) Spirit lamp
(v) Stop watch
(vi) Alcohol
(vii) Ether
(viii) Coton wool

* This is unnecessary in the hot weather months in India

17

PROCEDURES EMPLOYED IN INVESTIGATING HÆMORRHAGIC DISEASES

Introduction.—In the study of hæmorrhagic diseases and hæmorrhagic diatheses a number of procedures are commonly carried out that should be included in this series on hæmatological technique These are —

A Estimation of the coagulation time
B Estimation of the bleeding time
C Quantitative estimation of prothrombin in the blood or determination of prothrombin time
D Capillary resistance test
E Study of clot retraction
F Platelet count (*vide* p 27 et seq)

The study of clot retraction gives little additional information of value as it appears to be dependent entirely on the platelet count but as it provides confirmatory evidence we have included it *The estimation of blood calcium also has* some bearing on this subject but is essentially a biochemical test and will not be described here further though calcium deficiency could theoretically lead to a hæmorrhagic state in actual practice it does not appear to do so unless associated with other deficiencies

Discussion.—Briefly stated and essentially coagulation of the blood is dependent on fibrinogen ionized calcium prothrombin and thrombokinase (thromboplastin) The first three are present in the plasma while thrombokinase is derived from the disintegration of the platelets and damaged tissue the chain of events is as follows —

Prothrombin + ionized calcium + thrombokinase (thromboplastin) → thrombin
Thrombin + fibrinogen → fibrin
Fibrin + cellular elements of blood → blood clot
Clot + platelets → the retracted clot

(This leaves out of account the interaction between anti prothrombin prothrombin and thromboplastin substances Heparin—anti prothrombin—is a very real substance but whether it exists in the circulating blood in health does not seem definitely established so for the sake of brevity and we hope clarity we have not considered the part it may possibly play in preventing clotting in the circulating blood)

All the above substances are normally present in the blood a defect in any of them will cause a break in the continuity of the chain and will lead to hæmorrhage

Comment—The second method is more accurate as certain disturbing factors, *e g* tissue juice, are eliminated

B BLEEDING TIME

Apparatus required

(i) Pricking needle or blood gun (iii) Stop watch
(ii) Filter paper (iv) Sphygmomanometer

I DUKE'S METHOD

Clean the ear lobe or the dorsal surface of a finger first with alcohol and then with ether (The tip of the finger is not suitable for this test as it is horny in many cases) Prick the selected site fairly deeply with a blood gun or a pricking needle so that blood flows freely without squeezing Soak up the blood which comes out with a clean piece of filter paper without touching the skin The flow of the blood should be such that the first impression on the filter paper has a diameter of about half an inch Without squeezing or pressing the finger continue to soak up the blood with the filter paper every 30 seconds until the bleeding stops

The time from the first appearance of the blood until the cessation of bleeding is the bleeding time

Normal limits = 1 to 3 minutes

II VENOUS PRESSURE METHOD (Ivy *et al* 1935)

Put the cuff of a sphygmomanometer around the arm above the elbow as is done in taking blood pressure Select a portion of the forearm below the elbow and clean it with alcohol and ether Raise the pressure and maintain it at 40 mm of mercury which is sufficient to check any venous return Prick sharply up to a depth of about 1/10th inch at a point free from any superficial veins so that blood will flow freely Soak up the drop of blood every 30 seconds as in method I until the bleeding stops

This method is superior to method I as the capillaries are kept wide open and their tone eliminated also the area selected for puncture is uniformly thin For these reasons more consistent results are obtained with this method than with method I

Normal limits = 2 to 4 minutes

C QUANTITATIVE DETERMINATION OF PROTHROMBIN IN BLOOD PROTHROMBIN TIME

Apparatus required

(i) Ten c cm record syringe (iv) Water bath with a thermometer
(ii) Centrifuge tubes (v) Capillary pipettes with teats
(iii) Small test tubes in a rack (vi) Stop watch

Technique —Clean a finger or an ear lobe first with alcohol and then with ether Puncture sharply and fairly deeply with a blood gun or a pricking needle so that blood will flow freely without the necessity of squeezing Discard the first two drops of blood and then fill a capillary tube up to three fourths of its total length with blood (the capillary tube will fill by capillary attraction when held against the drop of blood) Note the time by the stop watch when the blood first enters the capillary tube Seal the free ends of the tube in a flame and put it in warm water (37 C) in a beaker Repeat the process with two other tubes noting the time of the commencement in each case

After one minute take out the first tube gently break off a portion of the tube from one end and repeat this every 15 seconds until a thin line of unbroken coagulum is seen stretched between the two broken ends note the time The difference between the two times by the stop watch is the coagulation time Repeat the procedure with the other two tubes breaking them at intervals of 15 seconds and find out the coagulation time for each tube

Take the mean of the three readings as the coagulation time

Normal time limits = 1 to 3 minutes

II Method of Lee and White (modified) with venous blood

Apparatus and reagents required (additional)

(i) An all glass 5 c cm syringe with a needle of fairly large bore
(ii) Small paraffined test tubes ⅜ inch in diameter with rubber corks in a rack
(iii) Normal saline
(iv) Hard paraffin in a metal or porcelain bowl

Prepare a water bath at 37 C place the paraffined tubes ready in the bath in an upright position

Collect 5 c cm of blood from a vein in an all glass syringe immediately after washing it out with normal saline *Note the time by the stop watch when the blood enters the syringe*

Take out the needle from the syringe and put exactly 1 c cm of blood into each of the three tubes Cork the tubes with rubber corks and allow them to stand in the water bath Wait for 3 minutes and then every 15 seconds in rotation take a tube and tilt the contents to see if the blood has set Continue doing this until the blood is found to have set so that the tube can be inverted without spilling any blood note the time

Normal time limits = 3 to 6 minutes

Preparing paraffin tubes —Melt some paraffin in a clean vessel Place a test tube with a glass funnel which has been previously warmed in a rack Pour the melted paraffin through the funnel into the tube up to about two thirds of its length Remove the funnel and pour back the excess paraffin from the tube into the vessel Immerse the tube in cold water taking care that no water enters the tube when the test tube is cold there will be a thin coating of paraffin inside the tube

Repeat the experiments with the other two tubes Take the mean of the three readings this gives the prothrombin time

Normal = 15 to 25 seconds

[More recently we have determined the prothrombin time in a few cases by using Russell viper venom alone and with ovolecithin added to it in the proportion recommended by Witts and Hobson (1940) With other workers we have found that the addition of ovolecithin shortens the coagulation time and the results are more uniform

Normal prothrombin time with ovolecithin = 10 to 15 seconds]

D HESS CAPILLARY RESISTANCE TEST

Apparatus required

(i) Sphygmomanometer
(ii) Stethoscope
(iii) Hand lens
(iv) Skin marking pencil

Place the cuff of a sphygmomanometer on the arm above the elbow as in taking the blood pressure

Determine the systolic and diastolic pressures release the pressure With a skin (or glass) pencil mark any purpuric spots that may be present on the forearm

— Raise the pressure to a point midway between the systolic and the diastolic and maintain it at this point for 5 minutes Remove the armlet and examine the forearm in a strong light and if necessary with a hand lens for purpuric spots (other than those already marked) A positive result indicating decreased capillary resistance is shown by the presence of a number of small fresh purpuric spots distributed over the forearm below the point at which the armlet was applied

The process should be repeated on the other arm

E CLOT RETRACTION STUDY

When blood coagulates a clot is formed which after a time contracts and expresses the serum the clot itself then shrinks and becomes hard Retraction of the clot as it is called varies directly with the number of platelets in the circulation but is independent of the coagulation time with a low platelet count 60 000 per c mm or below it takes over 24 hours for the clot to separate and the clot is soft and friable

Apparatus required

(i) Same as for collecting blood
(ii) Graduated 10 c cm centrifuge tubes each fitted with a cork having a hole in the centre
(iii) Glass rods with flanges about the size of a silver 4 anna piece half an inch from the end
(iv) Test tube rack

Method of Macfarlane —Collect blood from the patient in dry syringe (*vide* p 1)

Solutions required

(i) Sodium oxalate solution dissolve 1 34 gm of chemically pure anhydrous sodium oxalate in 100 c cm of distilled water

(ii) Calcium chloride solution dissolve 0 275 gm of chemically pure anhydrous calcium chloride in 100 c cm of distilled water Check the strength of the solution by titration against silver nitrate solution

(iii) Thromboplastin solution

Preparation of thromboplastin solution

Satisfactory results will be obtained with either of the following substances —

(*a*) From rabbit s brain Dissolve 0 3 c cm of dehydrated* rabbit s brain in 5 c cm of normal saline containing 0 1 c cm of sodium oxalate solution incubate at 45 C for 10 minutes and finally centrifuge at a low speed for 5 minutes Pipette off the milky supernatant fluid and keep the solution in a cold incubator floating a little toluene on the top of the solution The solution will keep for more than a month

(*b*) From Russell s viper venom *This is the method of choice*

A 1 in 20 000 solution of Russell s viper venom gives a potent thromboplastic solution In the dried state the venom maintains its full potency indefinitely kept in a refrigerator with toluene the solution also retains full potency for more than a month (Iyengar *et al* 1941)

Technique —A slightly modified Quick s method is followed —

Put 0 5 c cm of sodium oxalate solution into a centrifuge tube

Collect blood in a dry syringe (*vide* p 2) and put 4 5 c cm into the centrifuge tube containing oxalate solution mix well

Centrifuge until the plasma completely separates carefully pipette of the supernatant plasma and put it into another dry sterilized tube Put exactly 0 2 c cm of plasma into each of the three tubes marked A B and C and place them in the water bath at 37 C

Take out one tube at a time and to each add exactly 0 2 c cm of thromboplastin solution and 0 2 c cm of calcium chloride solution

Note the time when the calcium chloride solution is added shake gently keeping the tubes in the water bath and again note the time when the clot first appears

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* Dehydrating the brain Remove all the blood vessels and then macerate well in a glass mortar Add acetone and macerate again Decant off the acetone add some fresh acetone macerate and again decant off the excess of acetone Continue doing this until the whole thing is converted into granular powder keep in a cold incubator In the dried state its activity is retained for a long time

In different forms of anæmia this curve will vary in shape its median will move to the right or to the left and it may be of the normal height or flatter than normal

These variations in the curve can be expressed in figures The median can be given in terms of micron but it is more usual to give the mean corpuscular diameter (MCD) also in micron the flatness of the curve is shown by the standard deviation (σ) of the diameters of the cells from the mean diameter or more accurately by the co-efficient of variation (v) and also by the percentage microcytosis and macrocytosis These data can all be calculated from the Price Jones curve

The measurement of cell volume percentage and calculation of the mean corpuscular volume (MCV) have been described already (p 29 et seq) this MCV gives the volume of the cells but not their diameter Further it is a mean measurement and from it one gets no idea of the range of size of the individual cells it is therefore more limited in its application than the Price Jones curve

Neither measurement however alone will give information on the thickness of the cells but this can be calculated from MCV and MCD The method of making these calculations will be described later

The technique of the measurement of cell diameters —The technique described below is a modification of Hynes and Martin s method of measurement of red cell diameters

The images of the cell are projected on the vertical ground glass screen of a Bausch and Lomb euscope at a magnification of 2 000 and the measurements are made with the help of celluloid protractor on which a eries of circles with diameters increasing by the equivalent of 0 25μ have been drawn

Apparatus equ red

(i) A Bausch and Lomb euscope with projection screen
( ) A mechanical feed arc lamp with cond nser working 4 5 amperes
(i) A glass contain r filled with distilled water for cooling the b am of light from th arc lamp (filter)
(iv) A microscope with oil immersion objective and eye p ec to giv a magnification of 2 000 on the projection screen of the euscope
(v) A stage micrometer scale (Zeiss) with divisionns 10 apart
(vi) A celluloid cm /mm scale
(vii) Celluloid protractor for measuring the cells (v i)

Preparing the celluloid protractor —At a magnification of 2 000 1μ corresponds to 2 mm and 0 25μ to 0 5 mm The circles on the protractor are drawn with diameters increasing by the equivalent of 0 25μ (0 5 m m) i e the radii of the circles increa e by 0 25 mm The measurements of the radii of the series of circles are best obtained from a diagonal scale drawn to give measurements in multiples of 0 25 mm

Put 5 c cm of blood into the centrifuge tube Put in the cork with the glass rod passed through the hole in the centre of the cork See that the flanged end of the rod is near the bottom of the centrifuge tube (figure 27)

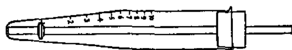


FIG 27 —Showing centrifuge tube with glass rod in position

Place the tube in an upright position in the rack and put the rack into an incubator at 37 C

From one hour onwards examine the tube from time to time for clotting

After one hour of clotting remove the clot by drawing out the glass rod (the clot will adhere to the flanged end of the rod)

Note the amount of fluid in the tube

The amount of fluid in the tube divided by the amount of blood put in the tube and multiplied by 100 gives the percentage of retraction

Example —Suppose 5 c cm of blood was taken and 3 c cm of fluid was left after the removal of the clot

Retraction $= \frac{3}{5} \times 100 = 60$ per cent

Normal retraction = 48 to 64 per cent

The clot is also examined for firmness and friability

Clot retraction is low in thrombocytopenic purpura but is normal in hæmophilia

18

RED CELL DIAMETERS PRICE-JONES CURVE

Introduction —In the normal blood the red cells are not all exactly of the same size but in no instance does the cell size deviate very much from the mean However in some forms of anæmia the mean size of the red cells is much below the normal mean in others it is far above it But in the latter case there are nearly always some exceptionally small cells as well so that the picture presented is that of cells of a great range of different sizes This blood picture is best presented in the form of a curve the abscissa being the size and the ordinates the number of times that cells of the particular size have been encountered (i e the frequency) In the normal count the curve rises sharply from the base line at about 6 0μ to a peak at 7 25μ and then falls almost to the base line again at 8 50μ This graph is usually known as a Price Jones curve In the normal individual the shape of the curve is more constant than the mean diameter of the cells which in a group of individuals will vary within but rarely fall outside the range 6 7μ to 7 8μ

Price Jones calculated what he called ideal maximum and minimum normal curves (Price Jones 1933)

area The magnification of the apparatus is adjusted to be 2 000 by using a suitable oil immersion objective and eye piece and varying the length of the draw tube of the microscope [A Zeiss microscope with objective 90 and a Leitz eye piece 15× with a draw tube length of 154 mm gives a magnification of 2 000 on the projection screen of the particular euscope used in our laboratory]

To find the magnification the distance between the images of two lines of the scale is measured with the celluloid cm /mm scale and at a magnification of 2 000 the images of the lines of the scale are 2 cm apart (2 cm = 20 000μ) [If the different components of the apparatus are kept fixed after the above adjustments the measurements can be carried out without repeating these adjustments every time]

Measuring the cells —The micrometer scale is next removed the blood film stained with a Romanowsky stain and counter stained with 1 per cent aqueous eosin solution is placed under the oil immersion objective and the image of the cells is focused on the ground glass screen of the euscope Only those images of the cells that fall within the reduced field are measured The celluloid protractor with the series of graduated circles is superimposed on the image of the corpuscles to find the circle that fits the image of each cell Each cell is measured to the nearest 0 25 The measurement of circular corpuscles is quite easy In the case of the irregular shaped corpuscles a circle is found such that the area of the corpuscle falling outside the circle is about the same as the area of the circle unfilled by the image of the corpuscle (*see* figure 30) Five hundred cells are measured from different parts of the blood film It is better to avoid the tail end and the thick parts of the smear as in the former the cells are excessively spread out and in the latter the cells overlap and are not well spread

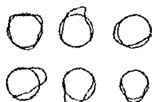
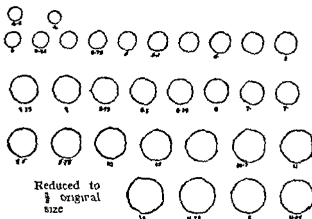


FIG 30

Recording the results —By this method 500 cells can be measured in about 40 to 90 minutes the time depending on the size of the cells and the degree of poikilocytosis the larger and more irregular the cells the longer is the time required An assistant is needed to note down the measurements and record the number of cells counted with a counting machine of the type of a Veeder counter

From the figures obtained the mean diameter standard deviation and coefficient of variation are calculated A Price-Jones curve is drawn by plotting the figures on graph paper The degree of microcytosis and macrocytosis can also be determined from this curve by comparison with the maximum and minimum ideal curves the overlapping of a curve beyond the minimum ideal curve on the low side and the maximum curve on the high side indicates the degree of microcytosis and macrocytosis respectively

The circles are drawn in Indian ink with very fine bow pen compasses on a celluloid sheet with radii increasing from 4 mm to 12 mm by steps of 0 25 mm so as to give measurements *corresponding to 4μ to 12μ* diameter Circles smaller and larger than the above are drawn on another sheet The first sheet is generally sufficient for routine work the second is needed very exceptionally (see figure 28)



Reduced to ⅓ original size

FIG 28

Assembling and adjusting the apparatus.—Figure 29 shows the euscope with microscope and arc lamp assembled The concentrated beam of light from the arc lamp cooled by passage through water in the filter is focussed on to the *concave* mirror of the microscope which reflects it through the condenser the objective and the eye piece of the microscope The total reflexion prism of the euscope placed over the ocular reflects the light on to the opaque screen of the euscope The condenser is fully raised up and with the high power objective turned on a bright beam of light is focussed on the opaque screen After the illumination has been adjusted the micrometer scale is placed under the obective of the microscope and the image of the scale is focussed on the opaque screen first with the low power and finally the oil immersion objective A final adjustment of light is made to give the maximum illumination The opaque screen is now moved off and the image is focussed on to the ground glass projection screen of the euscope By moving the microscope and/or the prism the image of the scale is so projected that the lines of the scale are vertical parallel and entirely free from spherical aberration at least within an area of about 4 inches by 3 inches marked out around the centre of the viewing screen

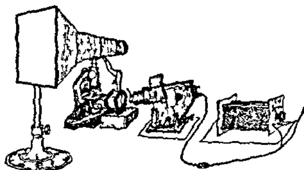


FIG 29

That there is no spherical abberation within this area can be shown by noting that the distances between the images of the lines are the same in all parts of this

From the mean corpuscular diameter (MCD) and the mean corpuscular volume (*vide* p 31) the mean corpuscular average thickness (MCAT) can be calculated by means of the following formula —

$$MCAT = \frac{MCV}{\pi r^2}\mu = \frac{MCV}{\pi\left(\frac{MCD}{2}\right)^2}\mu$$

Example—Let 90 cu μ be the mean corpuscular volume (MCV) and 6.52μ the mean corpuscular diameter (MCD)

$$\text{Mean corpuscular average thickness (MCAT)} = \frac{90}{\pi\left(\frac{6.52}{2}\right)^2}\mu$$

$$= \frac{90}{3.14 \times (3.26)^2}\mu = 2.69$$

The normal range of MCAT is from 1.7 to 2.5μ (Price Jones Vaughan and Goddard quoted by Whitby and Britton 1939)

In order to calculate the percentages of microcytosis and macrocytosis it is necessary to have ideal minimum and maximum curves for the population in which the investigations are being carried out Price-Jones calculated the ideal curves for subjects in Great Britain Working on similar lines we (Napier Sen Gupta and Chandra Sekar 1941) have worked out two similar curves Two smoothed curves are shown in figure 31 and the expected frequency distributions in the ideal minimum and maximum curves are given in table XVII

To arrive at the percentage microcytosis and macrocytosis the number of cells of each size (below 6.9μ) in excess of the number of that particular size that appear in the minimum ideal curve are summed this number divided by 5 to reduce it to a percentage is the percentage microcytosis Similarly the number of cells of each size (above 7.8μ) in excess of the number of that particular size that appear in the maximum ideal curve are summed and divided by 5 to obtain the percentage macrocytosis (*vide* table XVII)

Normals

	Number of observations	Mean corpuscular diameter (MCD)			Standard deviation (σ)μ			Co efficient of variation (v)		
		Max	Min	Mean	Max	Min	Mean	Max	Min	Mean
London (Price Jones 1933)	100	7.718	6.686	7.202	0.500	0.400	0.487	7.30	5.30	6.326
Calcutta (Napier Sen Gupta and Chandra Sekar 1941)	25	7.750	6.937	7.344	0.613	0.341	0.492	8.74	4.66	6.697

Method of calculating—The following figures show the method of calculating the mean cell diameter (MCD) the standard deviation (σ) and the co efficient of variation (v)

6 5μ the median has been taken as the arbitrary mean

TABLE XVI

Diameters in microns	Number of corpuscles in each class (f)	Deviation (d) of each class from arb mean in 0 25μ i e the class interval	f x d		f x d
5 25	4	− 5	− 20		100
5 50	18	− 4	− 72		288
5 75	34	− 3	− 10		306
6 00	67	− 2	− 134		268
6 5	74	− 1	− 74		74
6 50	107	0		− 402	0
6 75	70	+ 1	+ 70		70
7 00	50	+ 2	+ 100		200
7 25	46	+ 3	+ 138		414
7 50	18	+ 4	+ 72		288
7 75	5	+ 5	+ 25		1 5
8 00	4	+ 6	+ 24		144
8 25	2	+ 7	+ 14		98
8 50	1	+ 8	+ 8		64
				+ 451	
	500			+ 49	2 439

n = total number of observations = sum of frequencies (f) = 500

Σ f d = sum (Σ) of the product of the frequencies (f) and the degrees of deviation (d) from the mean = +49

Σ f d^2 = sum of the product of the frequencies (f) and the squares of the deviations (d^2)

Class interval = 0 25μ

Mean corpuscular diameter (MCD) = arbitrary mean + $\left(\frac{\Sigma \text{ f d}}{n} \times \text{class interval}\right)$

$= 6 5 + \left(\frac{49}{500} \times 0 25\right)\mu = 6 5245\mu$

Standard deviation $(\sigma) = \sqrt{\frac{\Sigma \text{ f d}^2}{n} - \left(\frac{\Sigma \text{ f d}}{n}\right)^2} \times$ class interval $= \sqrt{\frac{2439}{500} - \left(\frac{49}{500}\right)^2}$

$\times 0 25\mu = \sqrt{4 878 - (0 098)^2} \times 0 25\mu = \sqrt{4 878 - 0 0096} \times 0 25\mu = \sqrt{4 868} \times 0 25\mu$

$= 2 206 \times 0 25\mu = 0 551\mu$

Co efficient of variation $(v) = \frac{\sigma \times 100}{\text{MCD}} = \frac{0 551 \times 100}{6 525} = 8 4$ per cent

Significance of the findings.—It is not possible here to enter into any extensive discussion on the significance of the different findings but as a general rule it may be taken that in cases that have a high microcytosis percentage and a low MCV the anæmia is due to iron deficiency When such microcytosis is associated with a normal or even a high MCV it means that the cells are thicker than

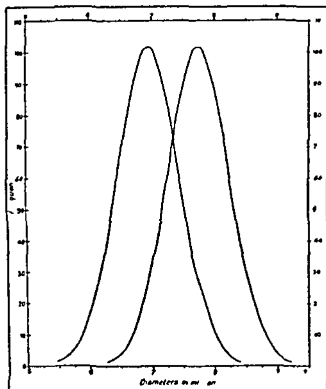


FIG 31.—Ideal maximum and minimum curves based on Indian data (Napier Sen Gupta and Chandra Sekar 1941)

normal and there is a tendency to spherocytosis such a condition is usually found in hæmolytic anæmias Pernicious anaemia shows a flat curve with a wide base usually a high percentage of macrocytes and a few microcytes whereas tropical macrocytic anæmia is usually associated with a moderately tall curve with an MCD slightly on the macrocytic side a few macrocytes and probably no microcytes

TABLE XVII

Measurement to nearest 0 25μ	Frequency distributions for ideal minimum curve MCD = 6 937μ σ = 0 4948μ	Frequency distributions for ideal maximum curve MCD = 7 750μ σ = 0 4948μ	Case X Count	Case X Excess of microcytes	Case X Excess of macrocytes	Case Y Count	Case Y Excess of microcytes	Case Y Excess of macrocytes
4 00						7	7	
4 25						2	2	
4 50			1	1		7	7	
4 75			0			8	8	
5 00			1	1		22	22	
5 25			1	1		30	30	
5 50	2		1			43	41	
5 75	6		6			38	37	
6 00	17		3			56	39	
6 25	39	1	11			45	6	
6 50	68	4	12			39		
6 75	93	14	29			41		
7 00	99	32	41			31		
7 25	82	61	70			47		
7 50	53	88	50			27		
7 75	27	100	40			9		
8 00	10	88	70			11		
8 25	3	61	40			9		
8 50	1	32	38		6	7		
8 75		14	35		21	10		
9 00		4	18		14	3		
9 25		1	16		15	5		4
9 50			8		8	3		3
9 75			1		1			
10 00			2		2			
10 25			1		1			
10 50			2		2			
10 75			1		1			
11 00			0					
11 25			1		1			
11 50			0					
11 75			0					
12 00			1		1			
			500		73	500	196	7

Case X

Percentage of microcytosis $= \frac{3 \times 100}{500} =$ 0 6 per cent

macrocytosis $= \frac{73 \times 100}{500} =$ 14 6 per cent

MCD = 7 791μ

$\sigma =$ 0 903μ

$v =$ 11 5 per cent

Case Y

Percentage of microcytosis $= \frac{194 \times 100}{500} =$ 38 8 per cent

macrocytosis $= \frac{7 \times 100}{500} =$ 1 4 per cent

MCD = 6 434μ

$\sigma =$ 1 07μ

$v =$ 16 6 per cent

Hæmagglutination and blood groups—The transfusion of blood requires the use of compatible blood that is blood in which the corpuscles of the donor are not agglutinated or hæmolysed by the plasma of the recipient (the patient) and the corpuscles of the recipient are not agglutinated or hæmolysed by the plasma of the donor

The phenomenon of hæmagglutination is due to the fact that there are in the blood of man iso-hæmagglutinogens in the red corpuscles and iso hæmagglutinins in the plasma there are two main iso hæmagglutinogens A and B that occur in the red cells and two corresponding agglutinins α (anti A) and β (anti B) in the plasma It is possible to divide mankind into four main groups according to the agglutinins and agglutinogens that are found in their plasma and red cells respectively Agglutinogens are present in the red cells of an infant from birth but specific agglutinins only make their appearance after some time Both increase in titre up to the age of puberty after which the titre of the agglutinogens remains stationary but that of the agglutinins gradually diminishes and is very low in old age Both the agglutinogens and agglutinins remain constant qualitatively throughout life These four groups are as follows —

Group (International nomenclature)	Agglutinogens in red cells	Agglutinins in plasma (or serum)
AB	A and B	none
A	A	β (anti B)
B	B	α (anti A)
O	none	α and β (anti A and anti B)

It is thus the agglutinogens in a person's red cells that give the name to his group though by simple deduction it is possible to ascertain his group from the agglutinins in his plasma (or serum) When A agglutinogen (in the red cells) comes in contact with α (anti A) agglutinin (in the plasma) agglutination takes place similarly agglutination takes place when B agglutinogen comes in contact with β (anti B) agglutinin

The arrangement of agglutinogens and agglutinins is always such that in any single individual auto hæmagglutination will not take place but the fact that in the human race there are four different groups of individuals precludes the indiscriminate mixing of blood of different individuals in vivo Provided bloods are of the same group they can usually be mixed with safety for example group AB blood can be given to an AB individual because neither the donor nor the recipient has α (anti A) or β (anti B) agglutinins in his plasma to act with the A and B agglutinogens in the red cells and cause agglutination but AB blood cannot be given—to an A individual because he will have β (anti B) agglutinins in his plasma

19
BLOOD GROUPING AND BLOOD TRANSFUSION

Historical—After Harvey's discovery of the circulation of the blood Sir Christopher Wren in 1650 injected opium beer etc into the veins of dogs using quills as cannulæ but the first transfusion of blood was given by Richard Lower in 1665 he transfused blood from one animal to another of the same species using dogs mostly as his experimental animals The credit however for performing the first transfusion in man is usually given to Denys de Montpellier physician to Louis XIV

After this transfusion was indiscriminately used as a curative agent in all diseases and in many cases animals were used as donors So many deaths followed this indiscriminate use of transfusion that the operation was banned by the Royal Society in England and by the Académie de Medecine in France and was condemned by the Pope as immoral Blood transfusion then fell into disuse as a therapeutic measure for about 150 years being revived in 1818 by James Blundell an obstetrician in Guy's Hospital For the first time he gave transfusion through a syringe in cases of post partum hæmorrhage and claimed immediate success He was of the opinion that transfusion by the new technique was a very feasible operation which after undergoing the usual ordeal of neglect opposition and ridicule will hereafter be admitted into general practice By this method whole blood was used for transfusion until 1835 when Bischoff first introduced defibrinated blood for this purpose and showed that it was safer as it obviated the dangers of coagulation However the transfusion of both whole and of the defibrinated blood still resulted in death very frequently and it was not until early in the present century that transfusion of blood was firmly established as a dependable therapeutic agent In 1900 Landsteiner an Austrian biochemist made the crucial discovery of the presence of iso agglutinins and iso agglutinogens in the blood On the basis of Landsteiner's discovery Jansky a Czecho slovakian and Moss an American independently showed (1907 08) that the blood of the human race belonged to one of four groups—I II III and IV—with this difference however that Jansky's groups I and IV corresponded respectively to groups IV and I of Moss groups II and III being identical in the two cases To do away with the confusion caused by the two different nomenclatures an international committee of the Health Organization of the League of Nations introduced a simplified and rational nomenclature for the four main blood groups which they called AB A B and O The international nomenclature of the blood groups and those of Jansky and Moss are shown in the table below

International nomenclature	Moss nomenclature	Jansky nomenclature
AB	I	IV
A	II	II
B	III	III
O	IV	I

Wash the red cells in the citrated saline tube three times with normal saline* Finally make a 5 per cent cell suspension in normal saline

The blood in the other tube is also centrifuged and the clear serum is then transferred to another labelled tube—this is an important detail neglect of which is liable to lead to serious mistakes and it must be emphasized that such mistakes may be fatal The name should at the same time be entered in a register and a serial number given which should also be written on the label

Apparatus and material required—

(i) Known high titre (anti A) and β (anti B) sera†
(ii) Known A and B cells—washed but not diluted
(iii) Glass slides 3 inch × 1 inch
(iv) Test tubes 2 × ½ preferably without lips
(v) Glass pencil
(vi) Capillary pipettes with rubber teats
(vii) Small solid glass rods with a diameter of not more than ⅛ inch and with the ends rounded off
(viii) Black and white tile (figure 32)
(ix) Hand lens
(x) Moist chamber (figure 33) This can be prepared by placing a piece of filter paper at the bottom of a petri-dish and keeping it wet with water Two glass rods of equal calibre are placed across the diameter of the petri-dish about 1 inch apart with a piece of glass rod of a smaller calibre inserted in between the two long glass rods to keep them apart It is then kept covered
(xi) The unknown washed 5 per cent red cell suspension in normal saline
(xii) The unknown serum

Procedure—To ascertain the group of an unknown sample of blood mix the unknown red cell separately with each known high titre serum which contain α (anti A) and β (anti B) agglutinins respectively wait for a few minutes and

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* The washing of the red cells is done in the following way —Centrifuge the blood in the citrated saline tube until the supernatant fluid is clear carefully pipette off the supernatant fluid add an equal volume of normal saline centrifuge again and again discard the supernatant fluid repeat the process a third time

† When doing grouping of blood for the first time known high titre and β sera should be obtained from any reliable institution where blood grouping is done as a routine Provided the whole process is carried out with the strictest aseptic precaution serum kept sealed in a refrigerator will undergo no changes for a year or more For subsequent use high titre α and β sera should be collected from time to time to replenish the stock from the subjects whose blood are grouped In places where blood grouping is done only occasionally the groups of the personnel of the laboratory hospital or dispensary should be ascertained so that in an emergency grouping of an unknown sample of blood can be carried out with α and β sera collected from among them even if the agglutinins are not present in high titre

A high titre serum is one which will agglutinate the appropriate red cells in a dilution of at least 1 in 10

In some countries dried high titre grouping sera α and β are now available such sera are supplied in powder form and are reconstituted by the addition of saline The advantage of these is that they are easy to send any distance they keep almost indefinitely and they can be reconstituted to form a hyper-concentrated solution

‡ Known A and B cells must always be collected freshly from persons known to belong to A and B groups for even when collected aseptically and kept in a refrigerator the cell hæmolyse in a few days and are not suitable for use There is also a danger in using stored cells as hæmagglutination has been found to occur when stored cells come in contact with bacteria especially of the corynebacterium group which may be present in the glassware used in performing the test (*Thomsen's hæmagglutination phenomenon*)

which will act with the B agglutinogen in the donor s red cells and agglutinate them to a B individual because of the α agglutinins or to an O individual because he has both α and β agglutinins

On the other hand in an O blood there are no agglutinogens so that such a blood can be given with safety to any person of his own group or as far as the donor s red cells are concerned to a person of any other group and for this reason such an individual is sometimes called a *universal donor* However in the plasma of the universal donor there are agglutinins both α (anti A) and β (anti B) and these are capable of acting on the appropriate agglutinogens in the recipient s red cells agglutinins are always weaker than the agglutinogens so that in practice if the blood is given slowly the agglutinins in the donor s plasma become diluted with the recipient s plasma and no agglutination of the recipient s red cells takes place unless as sometimes happens the agglutinins are present in exceptionally high concentration (high titre 1 in 32 or higher) a person who has agglutinin present in high titre is known as a *dangerous universal donor* and his blood should only be given to an individual of his own group

Now let us consider the position from the point of view of the recipient A B and O persons have in their plasma β (anti B) α (anti A) and both α and β agglutinins respectively and can therefore receive blood only from persons of their own groups that is persons who have not the corresponding agglutinogens in their red cells but an AB person has no agglutinins in his plasma so that he can receive red cells of any group and consequently an AB person is known as a *universal recipient* however his red cells contain both A and B agglutinogens and therefore if plasma from an A a B or an O person which happens to contain a high concentration of agglutinins is given his red cells may be agglutinated

For these reasons it is always essential that direct cross matching should be done before the blood of a universal donor is given to anyone except a person of his own group or the blood of anyone except a person of his own group is given to a universal recipient Further as there are a number of sub groups in the main blood groups—especially in groups A and AB and as an extra check against mistakes even when blood is given to a person of the same group it is advisable except in cases of great urgency to do a direct cross matching that is a matching of the donor plasma with the recipient s red cells and *vice versa*

A GROUPING

Collection of blood for grouping —Grouping of blood can be done with a drop of blood obtained by pricking a finger or the ear lobe but it is best done with washed red cells and the result checked by the cross identification of the agglutinins in the serum

Collect about 3 c cm of blood aseptically in a dry syringe put 2 c cm in a dry labelled test tube and 1 c cm in another labelled tube containing 3 to 4 c cm of citrated saline (1 5 per cent sodium citrate in normal saline) *Write the name and full description of the individual on the labels of the tubes*

Wash the red cells in the citrated saline tube three times with normal saline* Finally make a 5 per cent cell suspension in normal saline

The blood in the other tube is also centrifuged and the clear serum is then transferred to another labelled tube—this is an important detail neglect of which is liable to lead to serious mistakes and it must be emphasized that such mistakes may be fatal The name should at the same time be entered in a register and a serial number given which should also be written on the label

Apparatus and material required—

(i) Known high titre α (anti A) and β (anti B) sera†
(ii) Known A and B cells—washed but not diluted‡
(iii) Glass slides 3 inch × 1 inch
(iv) Test tubes 2 × ½" preferably without lips
(v) Glass pencil
(vi) Capillary pipettes with rubber teats
(vii) Small solid glass rods with a diameter of not more than ¼ inch and with the ends rounded off
(viii) Black and white tile (figure 32)
(ix) Hand lens
(x) Moist chamber (figure 33) This can be prepared by placing a piece of filter paper at the bottom of a petri-dish and keeping it wet with water Two glass rods of equal calibre are placed across the diameter of the petri-dish about 1 inch apart with a piece of glass rod of a smaller calibre inserted in between the two long glass rods to keep them apart It is then kept covered
(xi) The unknown washed 5 per cent red cell suspension in normal saline
(xii) The unknown serum

Procedure—To ascertain the group of an unknown sample of blood mix the unknown red cells separately with each known high titre serum which contain α (anti A) and β (anti B) agglutinins respectively wait for a few minutes and

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* The washing of the red cells is done in the following way —Centrifuge the blood in the citrated saline tube until the supernatant fluid is clear carefully pipette off the supernatant fluid add an equal volume of normal saline centrifuge again and again discard the supernatant fluid repeat the process a third time

† When doing grouping of blood for the first time known high titre α and β sera should be obtained from any reliable institution where blood grouping is done as a routine Provided the whole process is carried out with the strictest aseptic precaution serum kept sealed in a refrigerator will undergo no changes for a year or more For subsequent use high titre α and β sera should be collected from time to time to replenish the stock from the subjects whose bloods are grouped In places where blood grouping is done only occasionally the groups of the personnel of the laboratory hospital or dispensary should be ascertained so that in an emergency grouping of an unknown sample of blood can be carried out with α and β sera collected from among them even if the agglutinins are not present in high titre

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In some countries dried high titre grouping sera α and β are now available such sera are supplied in powder form and are reconstituted by the addition of saline The advantage of these is that they are easy to send any distance they keep almost indefinitely and they can be reconstituted to form a hyper-concentrated solution

‡ Known A and B cells must always be collected freshly from persons known to belong to A and B groups for even when collected aseptically and kept in a refrigerator the cells hæmolyse in a few days and are not suitable for use There is also a danger in using stored cells as hæmagglutination has been found to occur when stored cells come in contact with bacteria especially of the corynebacterium group which may be present in the glassware used in performing the test (*Thomsen's hæmagglutination phenomenon*)

then examine for the presence or absence of agglutination which will indicate the group of the unknown cell as shown below

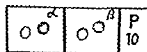


Fig 32

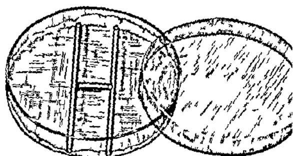


Fig 33

Example

	1	2	3	4
α (anti A) serum (from group B)+unknown cells	+	+	–	–
β (anti B) serum (from group A)+unknown cells	+	–	+	–
Group to which unknown blood belongs	AB	A	B	O

+denotes agglutination
–denotes no agglutination

For corroboration a further test with the unknown serum may be made The unknown serum is mixed with known A and B undiluted washed cells when one of the following combinations of reaction will occur indicating the group of the unknown serum —

Example

	1	2	3	4
Cells of group A+unknown serum	–	–	+	+
Cells of group B+unknown serum	–	+	–	+
Group to which the unknown belongs	AB	A	B	O

Technique

(1) *Tube method*

(a) *Test for agglutinogens in the cells* Place the test tubes 2 × ¼ in the first and second rows of a specially made rack With a glass pencil write the letters a 1 a 2 a 3 a 4 and so on on the first row of the tubes and the letters b 1 b 2 b 3 b 4 and so on on the second row of the tubes

Put one volume 0 04 c c of anti A serum into the tubes on the first row and one volume of anti B serum into the tubes of the second row and one volume of normal saline in all the tubes

The samples of blood to be tested are numbered 1 2 3 4 and so on a volume of red cell suspension from each sample of blood is put into the tubes

bearing identical numbers Each tube is rotated between the palms of the two hands to mix the contents and is capped to prevent evaporation The tubes are left for two hours

Reading the results —The tubes are picked up one by one the cap is removed and the tube is gently flicked with a finger to disperse the cell deposit from the bottom of the tube If definite agglutination is not visible to the naked eye in any tube the contents of that tube are examined microscopically The result are recorded as follows *

A *Visible to the naked eye*

C (complete)	Distinct clumps of cells
V (visual)	Weaker but distinct clumping

B *Seen under the microscope*

++	Very big clumps
+	Smaller clumps
(+)	Less than + clumps of 8 12 cell
W (Weak)	Small clumps of 4-6 cells
?	No definite clumps

Any reaction less than + is unlikely to be due to true hæmagglutination In such cases the cells must be tested again and the diagnosis confirmed by testing the serum

(*b*) *Test for agglutinins in serum* —The test is set up exactly in the same way as for tests for agglutinogens but in place of known anti A and anti B sera one volume of known A and B cells are put in each pair of tubes to which is added one volume of unknown serum and one volume of saline

The readings are taken after 2 hours and recorded as in (*a*) weaker reactions are more frequent and even a W reaction might be taken as a positive reaction

(ii) *Slide method (the method of choice)*

(*a*) *Test for agglutinogens in the cells* —Place a clean glass slide lengthwise on the white portion of the tile in front of you With a glass pencil put the mark across the slide half an inch from the right end of the slide and with another mark divide the remaining portion of the slide into two equal portions Write the letter α on the upper right hand corner of the extreme left portion the letter β on the upper right hand corner of the middle portion and the number of the unknown sample of blood on the extreme right hand portion of the slide (*see* figure 32)

Place a drop of anti A serum on the slide in the area marked α and a drop of anti B serum in the area marked β Place a drop of saline in both the areas

Rotate the tube containing the unknown cells between the palms of your hands to get a uniform suspension of the cells Withdraw a little cell suspension with a pipette and a put small drop of it by the side of but not in contact with the anti A serum and another drop by the side of anti B serum Mix each combination

* The signs used by the Blood Transfusion Research Committee (appointed by the Medical Research Council) have been followed

well with the glass rod using one end for each and then tilt the slide backwards and forwards to get an intimate mixture of the cells and the serum Place the slides on the glass rods of the petri dish over the moist filter paper and keep them covered (*see* figure 33)

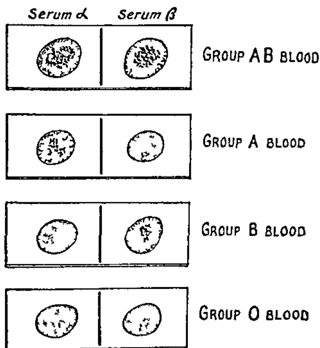


Fig 34

Examine for the presence of agglutination after 10 minutes In most cases clear cut results are obtained (*See* figure 34) but if there is any doubt about the presence of agglutination examine with a hand lens or under a microscope and record the results as in the tube method

(*b*) *Test for agglutinins in the serum* —The test with the unknown serum is made *exactly in the same way as for* tests for agglutinogens The unknown serum is mixed with known A and B washed cells and the readings are taken as in (*ia*)

For routine work we prefer the slide method to the tube method for only a very small amount of testing serum is required for each test and in most cases clear cut results are obtained in a much shorter time

Pseudo agglutination is occasionally seen in certain pathological states In hæmagglutination real clumping of the cells occurs whereas there is only rouleaux formation of the cells in pseudo agglutination If there is still any doubt add a drop of saline and shake the preparation gently by tilting the slide backwards and forwards when clumping due to hæmagglutination becomes marked but that due to rouleaux formation will often tend to disappear If there still any doubt the test should be repeated with a more diluted cell suspension when clear cut results will be obtained

Prozone phenomenon —*Very rarely undiluted serum will exhibit* a peculiar inhibitory property and agglutination will not occur but agglutination becomes quite evident when the serum is diluted this is known as the *prozone phenomenon*

B DIRECT CROSS MATCHING BEFORE TRANSFUSION

This should be done not only the first time but on each occasion that a donor gives blood to a particular recipient because instances have occurred when bloods that were compatible at the time of the first transfusion have on a subsequent occasion shown a degree of heterogeneity and some agglutination has resulted

Technique.—One drop of 5 per cent washed cell suspension of the donor is placed by the side of one drop of the serum of the recipient in one section of the slide and in the other one drop of serum of the donor by the side of one drop of 5 per cent washed cell suspension of the recipient. Each pair is mixed as in the original grouping procedure and in due course the two mixtures are examined for agglutination. If in neither case there is any agglutination this confirms the fact that the blood are compatible.

20

TRANSFUSION

Sources of blood

Donor's blood.—Healthy adults upto 50 years of age can donate 250 to 500 c cm of blood at a time once every two or three months according to the constitution of the donor.

Placental blood.—It has been suggested that the blood left in the placenta at delivery should be utilized for transfusion. Not only is it very difficult to ensure the sterility of this blood but any procedure that encourages the deprivation of the infant of even a drachm of the blood that is its due is in our opinion an immoral one. Moreover the blood collected from a single placenta is quite insufficient even for a small transfusion and is therefore not of great practical value.

Cadaver blood.—This has been used in Russia and in certain special circumstances the utilization of the blood of a body that has no further use for it seems justifiable. In a hot climate the difficulties would be so great that the method need not be discussed here.

Transfusion of different elements of blood

1 *Whole blood*.—Whole blood which acts as a complete tissue graft is ideal for transfusion where all the elements of blood are required. The whole operation of withdrawing and transfusing the requisite amount of blood must be completed within 3 minutes as otherwise the blood will clot in the syringe. The transfusion therefore has to be given very rapidly which may not be advisable. Even then the transfusion of whole blood demands a special apparatus or a team of trained workers with considerable dexterity when the ordinary syringe is used. For it is seldom practicable to use a syringe larger than 50 c cm and of these three will be required one to withdraw the blood and one to give it and the third one being washed out with sterile citrate saline and kept ready for use again.

2 *Blood to which some anti coagulant has been added*

(i) *Citrated blood*.—As an anti-coagulant various strengths of sodium citrate from 2 5 per cent to 3 8 per cent have been suggested the final strength of the sodium citrate in these mixtures is from 0 38 per cent to 0 5 per cent. We prefer to use 3 8 per cent sodium citrate arriving at a final dilution of 0 38 per cent with this strength the dilution of the blood is not great and it is easy to calculate the amount of sodium citrate solution required viz 10 c cm for each 100 c cm of the mixture.

Citrated blood can be used immediately or if collected with certain precautions it may be preserved* in a refrigerator between 4 to 6 C and used later ten days is usually considered the maximum safe storage time

A great advantage in using citrated blood is that *the time factor is unimportant* The flow can be regulated and if necessary it can be given by the drip method Transfusion of citrated blood is however often followed by a mild reaction

(ii) *The use of heparin as an anti coagulant*

(a) *In vitro* heparin may be used in place of sodium citrate solution 4 mg for each 100 c cm of blood is recommended the high cost of heparin limits its use

(b) *In vivo* 1 mg per kilogram body weight is given intravenously to the donor After the injection the coagulation time is greatly lengthened although this relative incoagulability of the blood persists for 30 to 60 minutes or more *it is recommended that the blood be collected from the donor 7 minutes after* the injection that the transfusion be given through a wide bore needle and that it be completed within 15 minutes Injection of such a blood is not injurious to the patient in any way and does not alter the coagulation time of his blood

Transfusion by this method can be given with a 50 or a 100 c cm syringe and no special apparatus is required

3 *Plasma or Serum*

Plasma —If the citrated blood is allowed to stand undisturbed in the refrigerator the corpuscles completely settle to the bottom of the bottle in two or three days time and leave the clear plasma at the top The plasma can now *be pipetted off without disturbing the corpuscles at the bottom*

Serum —If blood is collected without the addition of any anti coagulant the serum separates out in about 24 hours The serum can now be pipetted off *without disturbing the clot*

Thus separated the plasma or serum after being filtered can be used at once stored in a refrigerator for future use or dried by various processes and later redissolved and used The great advantages of dried plasma or serum are reduction in bulk and ease of storage as it is not necessary to keep dried plasma or serum in the cold Properly dried plasma or serum can be u ed for at least two years from the date of its collection Transfusion of plasma or serum is not usually *attended with any reaction it does not transmit malaria and grouping of the* blood is made unnecessary if the blood of several persons of different groups is mixed before or even after the plasma or serum is eparated They are ideal therapeutic agents to combat shock and are being very extensively used in the war as their small bulk facilitates transport

4 *Resuspended Corpuscles* —The concentrated corpuscles remaining at the bottom of the bottle after the plasma is separated off can be used for transfusion in suitable cases of anæmia According to Kolmer (1940) these red cells can be

* Following preservative is recommended (Mollison and Young 1941) for stored blood —

Sodium citrate 3 per cent	18 5 c cm
Glucose 30 per cent	1 85 c cm

For each 100 c cm of blood to be taken

used with satisfactory results up to about eight days after which their oxygen carrying capacity and other properties begin to diminish gradually The concentrated red corpuscles are diluted with glucose saline—to each 400 c cm of sedimented red cells 50 c cm of 1 1 per cent saline with 4 5 grammes of glucose is added so that the final strength of the glucose in the mixture 1 1 0 per cent The chances of a reaction following the transfusion of the concentrated red cells will be reduced if the leucocytes and platelets which form a white layer on the top of the red cells are discarded and if the mixture is filtered before administration (Macquaide and Mollison 1940)

5 *Defibrinated blood* —The use of defibrinated blood is now almost obsolete for the process of defibrination removes many vital properties of the blood and also renders it more toxic

Indications for transfusion

1 *After acute hæmorrhage* —To make good loss sustained by an acute hæmorrhage the transfusion of whole or citrated blood either preserved or fresh is the most effective The amount to be transfused will be dependent on the amount lost but anything less than 500 c cm is unlikely to be effective

2 *Shock* —From whatever causes In shock without hæmorrhage plasma or serum is better than whole blood two or more litres may be necessary

In shock with hæmorrhage in addition to plasma or serum transfusion of citrated blood 300 to 500 c cm depending on the amount lost should also be given

3 *As a prophylactic measure against shock* —In weak or exsanguinated patients even if their condition would not ordinarily demand transfusion this is often a very valuable procedure to prepare them for a surgical or obstetric operation blood or plasma is given according to the blood state of the patient

4 *In anæmia* —Blood transfusion is indicated to replace blood lost by hæmorrhage or destroyed by other processes to maintain life in the case of aplastic anæmia and/or to stimulate blood formation Whole blood citrated blood or resuspended corpuscles can be used In anæmia repeated small transfusions of 300 to 400 c cm are usually better than a single big transfusion of say 1 000 c cm in either case slow administration is to be recommended

5 *For supplying other deficit elements* viz platelets in thrombocytopenic purpura leucocytes in agranulocytosis or fibrin in some cases of clotting deficiency transfusion of whole blood will sometimes be effective

6 *For counteracting toxins or increasing passive immunity* —This is a procedure that was at one time in favour particularly in America the bloods of convalescents or of others who had received injections in order to raise the antitoxic value of their blood or to produce active immunity were used as specific transfusions in certain cases However the new chemotherapeutic agent introduced in the last few years have put this procedure into the background

Technique of collection and transfusion of blood

Apparatus and solutions required

(i) Sphygmomanometer
(ii) Stethoscope
(iii) Bottle for collection of blood (figure 35) *
(iv) Standard bottle for collection and transfusion of blood (Fig 36) *
(v) Potain s aspirator or a Higginson s syringe
(vi) Glass container with an opening at the bottom to serve as a reservoir for the blood (Fig 37)
(vii) Two moderate size serum syringe needles in a beaker containing liquid paraffin and a suitable adapter (figure 36) It is better to have two sizes as a smaller bore is required for giving than for drawing blood
(viii) Rubber pressure tubing
(ix) Surgical silk thread
(x) Pair of scissors
(xi) Two enamel bowls
(xii) Lifter
(xiii) Drum of sterilized dressings and towels
(xiv) Thermometer
(xv) Rubber cloth
(xvi) Measuring cylinder
(xvii) Examination couch or easy chair for the donor
(xviii) 3 8 per cent sodium citrate solution and normal saline both freshly prepared with pyrogenfree† distilled water and sterilized
(xix) Tincture of iodine
(xx) Tincture of benzoin
(xxi) Absolute alcohol
(xxii) Bowl of antiseptic lotion
(xxiii) Glass of water or some beverage to counteract any temporary psychological shock experienced by the donor

Additional apparatus if the transfusion is given by the open method

(i) A splint to immobilize the limb in which th transfu ion is given

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* A Potain s aspirating bottle makes a very useful receptacle for taking blood from a donor Pieces of pressure tubing are attached to the inlet and outlet taps to the other end of the inlet tube a record adapter is inserted ready to receive the needle and the other end of the outlet tube is attached to the Potain s syringe As an extra precaution to prevent micro-organisms passing into the bottle during the back stroke of th Potain s aspirator a piece of glass tubing containing a plug of sterile cotton wool may be inserted in the length of the pressure tubing

The Potain s aspirating bottle is graduated on its outer surface so that the amount of blood collected can be seen at a glance

The joints taps etc must be absolutely air tight

*There are many standardized bottles now in use which can be used for collecting storing and giving blood without transferring it the chances of contamination are thus reduced to a minimum The bottles are so made that for giving blood a transfusion tube with filter is inserted and is hung in an inverted position Such a bottle is described by Baily (1940) (figure 38) *See* also Hayes *et al* (1941)

† Water may be made pyrogen free in the following way —

In a clean glass still re-distil some freshly distilled water to which a little sulphuric acid and one or two crystals of potassium permanganate have been added to give it a faint pink colour If during the process of distillation the pink colour disappears from the water in the still a little more sulphuric acid and potassium permanganate must be added

The distillate is collected in a closed glass flask which has been previou ly prepared by rinsing first with a solution of potassium bichromate and sulphuric acid then washing out first with distilled water and then with pyrogen free water and finally sterilized by autoclaving

The pyrogen free water is sterilized in an autoclave and may be used for about three to four days

(iii) Dissecting instruments viz scalpel scissors probe forceps and an urism needl
(iv) Sterilized silk
(v) Curved needle and silkworm gut

Selection of donors

Strong healthy persons between the ages of 20 and 45 are the best donors The donor must be free from any infectious disease and his blood must always be examined for syphilis (Wassermann or Kahn reaction) He should be questioned about allergic manifestations e g asthma urticaria etc as a temporary sensitivity to allergens may be transmitted through transfusion Malaria in a donor is a special danger in this country especially if the recipient has never had malaria

Preferably the donor should be of the same group as the recipient but a safe universal donor group O (*vide* p 108) may be used for giving blood to people of all four groups and a universal recipient (group AB) may receive blood from donors of all four groups The following table shows the groups of suitable donors for different recipients —

If recipient belongs to group	Donor may be of group
AB	AB A B or O
A	A or O
B	B or O
O	O

In all cases the cells and serum of the recipient must be cross matched with the serum and cells of the selected donor even when the recipient and the donor are of the same group After a transfusion this cross matching of blood between the donor and the recipient must always be done again before another transfusion is given because the previous transfusion may produce some change in the blood of the recipient which will render it incompatible with the blood of a donor whose blood was found to be suitable before a transfusion was given

The same donor must never be used for the same recipient after an interval of more than ten days after the last transfusion on account of the dangers of anaphylactic like reactions that have been reported

A healthy donor of average size can given 500 c cm of blood once a month without any detrimental effect to his health After such bloodletting it takes seven days to make good the loss of blood but the compensatory mechanisms of the body are so efficient that whilst this deficit is being made up the donor will not be conscious of the loss in any way and will be capable of performing his full day s work as usual

Collection of blood

The donor should if possible fast for 2 to 3 hours before the blood is taken because some articles of diet though innocuous to the donor may produce allergic symptoms in the recipient

The donor should be made to lie comfortably on the couch and there should be sufficient light on the selected arm A sheet of rubber cloth should be placed under the arm

(i) *Collection in a Potain s aspirating bottle* (fig 35)

Put the requisite amount of 3 8 per cent sodium citrate solution (10 c cm for 90 c cm of blood to be taken or for every 100 c cm of the mixture) in the bottle from which the air is now partially exhausted by pumping with a Potain s aspirator syringe or with a reversed Higginson s syringe if the former is not available Fit a needle of medium calibre on to the tube attached to the inlet tap and test the negative pressure in the bottle by immersing the needle in sterilized saline and partially opening the stop cock in the inlet tap when the saline will be sucked into the bottle The bottle is now ready for use

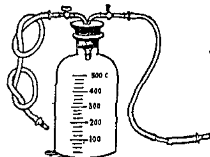


Fig 35 —Potain s aspirating bottle used for receiving blood

In the meantime an assistant has taken the systolic and diastolic pressures of the donor *The skin over the vein is sterilized with iodine and then swabbed* with alcohol to remove the stain of the iodine Raise the pressure to about the level of the systolic readings puncture the vein with the needle* and when it is in the vein open the stop cock in the inlet tap and lower the pressure to about the level of the diastolic reading when the blood will flow freely into the bottle The bottle is shaken gently so that the blood mixes thoroughly with the citrate solution and does not clot When the requisite amount of blood has been collected *let* out the air *from* the sphygmomanometer *close the stop-cock and then take* out the needle from the vein Apply digital pressure over the puncture for a minute or two seal with tincture of benzoin apply a sterile dressing and put on a firm bandage

(*ii*) Collection in a standard bottle (fig 36)

Put the requisite amount of sodium citrate solution (V S) or if the blood is to be stored a mixture of sodium citrate and glucose (p 112) in the bottle With the cap lightly fitted the bottle with the solution is sterilized in an autoclave When still very hot bring out the bottle from the sterilizer and screw the cap *tightly This will produce a quite good vacuum* in the bottle The bottle is now ready for use

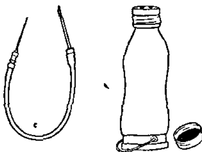


Fig 36
The tandard and bottle for collection of blood C —The cap with a piece of rubber inside
C T —The collecting tube with *needles N for the donor and N for* the bottle

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* If the vein is not prominent or the operator is inexperienced it is not advisable for him to attempt to puncture the vein with the needle alone but to attach it to a syringe a little blood is drawn into the syringe to make certain that the needle is in the vein the syringe is then detached from the needle to which the adapter in the end of the collecting tube is quickly fitted The vein seeker which is sometimes recommended achieves much the same end

Now proceed as in (i) using the collecting tube (fig 36) in place of the tube attached to the inlet tube of the Potain's bottle Puncture the vein with the needle (N_1) attached to one end of the collecting tube and when it is in the vein pierce the rubber through one of the two holes in the cap of the bottle (fig 36) with the stout needle (N_2) attached at the other end of the tube when the blood will flow freely into the bottle When the requisite amount of blood has been collected let out the air from the sphygmomanometer and take out the needle from the vein Seal and dress the puncture

After the collection of the blood give the donor a drink of cold water or milk or if the donor shows any signs of psychological shock some stimulant e g sal volatile or some suitable form of alcohol

Transfusion of blood

Make certain that all the apparatus for transfusion is ready and sterilized before starting the operation For the method of sterilizing the different parts see below

Select a good vein and arrange the patient comfortably on the bed with his arm resting on a pillow covered with rubber cloth

(i) *Transfusion with the ordinary apparatus* (fig 37)

Sterilize your hands and fit one end of a piece of sterilized rubber tubing to the bottom of the glass reservoir A record adapter to which is fitted a needle of small bore is now firmly fitted to a narrow tube about 2 inches long the other end of this tube is fitted by means of a glass connection to the end of the long rubber tube attached to the glass reservoir (figure 37) The glass and the rubber connections the rubber tubing with the reservoir glass tubing and the adapter are now firmly tied with silk If the transfusion is to be given very slowly the drip apparatus is incorporated at about the middle of the long rubber tube attached to the glass reservoir To the rubber tube a little above the drip apparatus is attached a pinch cock to regulate the flow of the transfused fluid

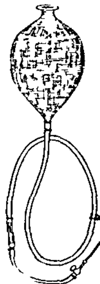


Fig 37.—Transfusion apparatus for giving blood or saline with the drip feed apparatus incorporated in the tube (in this figure the length of the tubing between the drip feed and the needle is shorter than it would be in practice)

Wash out the apparatus by pouring sterilized normal saline into the reservoir drive out the air bubbles in the tube by squeezing the rubber tubing and see that the saline flows freely through the needle, leaving a little about 10 c m of saline in the glass reservoir but when a drip apparatus is used manipulate the pinch cock above it so that the saline flows only in drops through the drip apparatus and consequently also at the same rate through the needle at the extreme end

The skin over the selected vein is sterilized with tincture of iodine and an assistant applies pressure with one hand just above the selected vein in order to make

it stand out prominently The operator now punctures the vein with a successful puncture the blood from the patient will flow back into the glass tubing but, when the pressure above the vein is released the flow is thereby reversed and the saline will pass into the vein (As in the case of the donor the puncture may first be made with the needle attached to a small syringe a little blood withdrawn the syringe detached from the needle and the adapter at the end of the rubber tubing from the reservoir fitted to the needle) When it is seen that the saline is passing into the vein the citrated blood in the bottle is gently shaken and then poured into the glass reservoir through a few layers of sterilized gauze placed on the top of it When most of the blood has been given a little warm saline is added to wash out the residual blood in the reservoir and the tube the needle is taken out and for a minute or so pressure is applied to the puncture which is then sealed with tincture of benzoin and a bandage applied over a sterile dressing

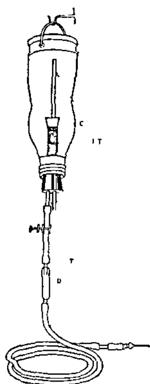


Fig 38

The standard bottle fitted with transfusion outfit
I T — Inlet tube for air
F — Filter for blood made of fine wire gauze
C — Rubber cork to hold the filter in position
C — Rubber cork which fits into the mouth of the bottle
I T. — Transfusion tube
S — Stopcock
D — Drip feed apparatus

(*ii*) *Transfusion with the standard apparatus* (*fig 38*)

When stored blood is used, the temperature is *slowly raised to 37°C by putting the bottle with* the blood in a vessel containing hot water the temperature of which must not be very high When the temperature has been raised to about 37°C the cap is taken out and the transfusion outfit is fitted into the bottle—the outer rubber cork will fit in nicely into the mouth of the bottle but as an extra precaution the cork is kept fixed to the neck of the bottle with adhesive plaster

The bottle is now inverted and with a little manipulation a continuous column of blood without any air bubble is made to fill up the whole length of the tube The pinchcock above the drip apparatus is manipulated so that the blood flows in drops through the drip apparatus The vein is punctured and the rate of flow is regulated by manipulation of the pinchcock When all the blood has been given the needle is taken out and the puncture sealed as in (*i*)

Rate of transfusion

The rate at which blood is transfused will depend entirely on the circumstances In a case of acute and serious blood loss a rapid transfusion is indicated 500 c cm being given in 10 to 15 minutes but in chronic conditions especially cases of sever anæmia the drip feed method by which 1 c cm per pound weight of *patient per*

hour is given or an even slower rate in which it takes 24 hours to give 500 c cm is preferable

The open method—The transfusion by the closed method is possible in all but extremely debilitated patients with very low blood pressure and collapsed veins in those cases transfusion by the open method is resorted to

The vein used for open transfusion is subsequently ligatured and for that reason and on account of the greater convenience of keeping the leg immobilized for a long time the great saphenous vein is usually chosen The leg is immobilized with a splint the vein is opened and a Rogers cannula which is used in place of the ordinary needle is inserted into the vein The cannula is kept in position with adhesive plaster With this technique blood can be given by the drip method at a very slow rate over a long period 24 hours or more without tiring the patient or interfering seriously with his movements in bed

Cleaning of the apparatus

As soon as the transfusion is finished separate all the parts of the apparatus wash in tap water and soak in water for several hours

The different parts are then cleaned in the following way —

Bottles—Thoroughly scrub the inside of the bottle with a stiff test tube brush wash in hot water

Needles—Clean with stillete wash with hydrogen peroxide and then with distilled water dry in alcohol and ether and keep in a vessel containing liquid paraffin

Other parts—Thoroughly clean all the parts of any vestige of blood with water using a stiff wire for the rubber tubing Next wash with dilute solution of soap and rinse in water to remove the soap Place all the parts in a large pan containing 0 1 per cent NaoH and boil for 5 minutes Transfer to another bowl and wash first with distilled water and finally with pyrogen free water Allow to dry re assemble thet different parts and pack for sterilising

Sterilization

(i) The different parts of the transfusion set packed in a cloth bag and the glasswares e g bottle for collection of blood and the glass reservoir for transfused blood are sterilized in an autoclave at 120 C for half an hour

Dressings are also sterilized in the same way

(ii) Needles—by boiling in liquid paraffin for about 10 minutes

(iii) Cutting instruments—in strong lysol lotion and washing in sterilized distilled water before use

Reactions after transfusions

Transfusion of citrated blood is very often accompanied by slight reactions which are sometimes due to pyrogenic substances present in the apparatus and in the distilled water used for preparing solutions There is seldom any reaction after transfusion of plasma alone

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Reactions after transfusion may be divided into three groups —

(i) due to defects in the donor

(ii) due to technical causes and

(iii) due to autogenous causes in the recipient

These reactions will therefore to some extent be avoided by

(i) careful selection of donors particularly avoiding allergic subjects

(ii) following carefully the instructions given above and giving particular attention to the following—washing all the apparatus with pyrogenfree normal or citrated saline so that no trace of distilled water is left in any of them and avoiding excessive shaking overheating and rapid administration of the blood and

(iii) taking action according to the circumstances as follows —

Autogenous causes in the recipient	*Remedies*
(a) Susceptibility of the patient to shock	Lower the head end of the bed cover with blanket and apply heat to the extremities If required 0 5 c cm of adrenalin chloride may be given hypodermically
(b) Allergy	Adrenalin chloride 0 5 c cm hypodermically calcium by the mouth and by injection and calamine lotion locally in the case of urticaria
(c) Lysis of recipient s blood (generally seen in cases of hæmolytic anæmia)	An alkaline mixture should be taken before the transfusion and the transfusion should be given very slowly

Still another kind of reaction may occur in a small number of cases due to the difference in the Rh agglutinogen in the recipient and the donor It has now been definitely established that even when the donor and the recipient belong to the same ABO group lysis of the donor s blood may occur after the second and/or subsequent transfusions if the recipient is Rh negative and the donors are Rh positive Symptoms simulating those seen after a mismatched transfusion e g respiratory distress pain in the back vomiting may appear within a few minutes and later blood may appear in the urine If the symptoms appear during the course of a transfusion the transfusion should be stopped at once In all such cases every effort must be made to alkalinise the urine and to promote simple diuresis by administration of big doses of Sodium citrate e g two drachms at once and one ounce during the following 24 hours and subsequently till the urine is free of blood pigments In very severe cases 150 c c of 3 per cent Sodium citrate solution should be given intravenously at once which is to be followed by 450 c c of 3 per cent Sodium citrate solution mixed with 2400 c c of 5 per cent glucose and given very slowly by drip during the next 24 hour

Prompt transfusion of 200 300 c cm of compatible blood from Rh negative donor is reported to give excellent results

21

THE INVESTIGATION OF A CASE AND THE REPORTING OF RESULTS

The question that we are frequently asked is what do you consider the minimum examinations that should be carried out in a case of anæmia? We should be forced to reply that with the exception of those in section 17 which apply particularly in hæmorrhagic diseases *at least* all the examinations that have been described in this little book should be made for the proper investigation of a case

In actual practice nearly all the blood examinations can be carried out from a single specimen of blood and do not take a good technician more than an hour if the Price Jones curve is excluded A certain amount of time can be saved by omitting the Arneth count the fragility test and the enumeration of thrombocytes as there are many circumstances in which these tests will give little information of practical value This leaves the hæmoglobin estimation the total red and white cell counts the reticulocyte count cell volume percentage estimation (hæmatocrit) and sedimentation rate van den Bergh s reaction and white cell differential count to be done as a minimum routine procedure in every case

The findings will indicate whether these examinations should be supplemented Sternal puncture gives very valuable additional information but it must not be considered as a short cut to diagnosis for it will seldom be of any value without a complete blood count and further the identification of the various cells is a very skilled work that necessitates considerable experience

There will be occasions when electric current is not available and in these circumstances it will not be possible to estimate the cell volume percentage satisfactorily The mean corpuscular hæmoglobin (MCH) however usually runs more-or less parallel with the mean corpuscular volume (MCV) and it will be advisable to base the diagnosis on the mean corpuscular hæmoglobin alone The determination of the cell diameter by clinical halometres though very easy and simple is in our opinion very inaccurate specially in pathological conditions and should never be employed

The inclusion of the erythrocyte sedimentation rate (ESR) in the minimum procedures might be questioned but if a Wintrobe s tube is used for cell volume estimation the ESR requires no additional apparatus and entails no extra labour except the taking of a reading at the end of an hour and the information it gives is often useful

Gastric analysis is important when the question of pernicious anæmia arises and in some cases of microcytic anæmia of doubtful ætiology but except in these cases and as a general procedure in investigating the function of the gastro intestinal tract dysfunction of which is so often an ætiological factor in anæmia in India we would not give it a high place in the order of importance in the investigation of cases of anæmia in this country unless there are associated gastro-intestinal symptoms

For the complete investigation of the ætiological factors many other examinations will have to be carried out e g the urine for albumin blood and ova the stools for protozoa helminths and pathogenic bacteria for fat content and for the presence of occult blood and the blood for malaria aldehyde test blood grouping the Wassermann reaction cholesterol albumin/globulin ratio etc

Controlling progress—Circumstances will usually dictate how often blood examinations should be carried out during treatment but there are certain points which we will consider here

In the first place when a seriously ill patient is admitted into hospital he is often suffering from some degree of hæmo concentration and a second examination made a few days after admission may show a marked drop in hæmoglobin percentage whereas the patient s condition may have improved considerably If allowance is not made for this fact the effect of the first treatment that is given may be misjudged and we have always adopted the practice of making a second examination before prescribing specific treatment (except in urgent cases where an immediate blood transfusion is indicated)

Further there are many nutritional anæmias in India in which there is a steady improvement directly the patient is put to bed and given a good diet Allowance must also be made for this fact if the value of any special form of treatment is being estimated and in such circumstances at least two weeks of rest and diet should be allowed before the specific treatment is started

We make a practice of repeating the blood examination including hæmoglobin percentage red cell count reticulocytes cell volume percentage van den Bergh s reaction and ESR and examination of the film for abnormal cells once a week or earlier if a set back is suspected or if the treatment is to be changed In special cases other examinations such as the platelet and leucocyte counts are also repeated

When any specific form of treatment is given a reticulocyte count is done daily from the 4th until about the 12th day or in a case where there is a reticulocyte crisis until the reticulocyte percentage has fallen to its previous level

The printed forms that we use in the School are shown below There is quite possibly room for improvement in these forms and we have modified them from time to time but we find that without being cumbersome they are sufficient for our purpose

Form 1 is the ordinary report form which is sent to the ward and attached to the notes size 8½ by 6½ inches

Form 2 is the sternal puncture report form similarly sent to the ward and attached to the notes a résume of this is entered on the reverse of form 3 size 8½ by 5½ inches

Form 3 is the card which we keep in the laboratory The important data on which progress is judged are entered on the face of the card and other data and records of examination that are not usually repeated on the reverse size 8½ by 6½ inches—stiff card

FORM I

S. T. M. ANÆMIA DEPT.

BLOOD REPORT

Name *Sex* *Age* *Ward* *Bed*

Hæmoglobin	gm. per 100 c.cm.
Red blood cells	per c.mm.
Reticulocytes	per cent
Cell volume	per cent
Mean corp. volume	cu. μ
Mean corp. hæmoglobin	γγ
Mean corp. hæmoglobin conc.	per cent
White blood cells	per c.mm.

	per cent	per c.mm.
Neutrophils		
Lymphocytes		
Monocytes		
Eosinophils		
Basophils		

Arneth count I II III IV V Weighted mean *Blood group*

Abnormal cells

Platelets— per c.mm.

Coagulation time *Bleeding time*

Fragility of red cells *Prothrombin time*
Capillary resistance test

Sedimentation rate (1 hour) {Observed
Corrected—

Van den Bergh's test {Direct
Indirect} mg. per 100 c.cm.

Opinion

Date

For the complete investigation of the ætiological factors many other examinations will have to be carried out e g the urine for albumin blood and ova the stools for protozoa helminths and pathogenic bacteria for fat content and for the presence of occult blood and the blood for malaria aldehyde test blood grouping the Wassermann reaction cholesterol albumin/globulin ratio etc

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FORM 3

ANÆMIA CARD

S T M — ANÆMIA DEPT

Name — *Sex* — *Age* — *Ward* — *Bed*

Date	Treatment and diet	Hb	RBC	Ret	CV	MCV	MCH	MCHC	Leucocytes						v d B	ESR	Abnormal cell
									Total	N	L	M	E	B			

FORM 2

S T M ANÆMIA DEPT

STERNAL PUNCTURE REPORT

Name *Age* *Ward* *Bed*

Total nucleated cells Reticulocytes

Endothelial cells
Red cell series
Pro erythroblast

Megaloblast { A, B, C

Normoblast { A, B, C

White cell series
Granular series—
Myeloblast
Pre myelocyte
Neutro myelocyte { A, B
meta myelocyte
band
segmented
Eosino myelocyte
meta myelocyte
band
segmented
Basophils

Non granular series—
Lymphoblast
Lymphocyte { A, B
Plasma cell
Monoblast
Pre monocyte
Monocytes
Megakaryocyte
Undifferentiated
Parasites

Date

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FORM 3 (reverse)

Blood

Platelets — Fragility / Arneth count — Coagn time I II III IV V — Bleeding time Wt mean — Cap res W R — Prothrombin time / Blood group

Sternal puncture

Total — Gran — Non gran — Red — Pro Erbt — Mgbt A B C — Normo A B C — Ret

Spleen puncture —

Gastric analysis — F ¼ ½ ¾ 1 1¼ 1½ 1¾ 2 2¼ 2½ 3 hours

Price Jones curve

Peptic d gest on

Mean corpuscular diam (MCD)
Standard deviation (*S d*)
Coefficient variation (*v*)
Per cent macrocytosis
microcytosis

Urine

Stool { Ova / Protozoa / Occult blood

Other data

Spleen

Liver

Weight

Clinical notes

Epidemiological data

Economic status

Dietary habits

Re idence—malarious or not

Occupation

Family h tory

ERRATA

Page	Line	For	Read
6	13	nmenclature	nomenclature
14	20	dilnted	diluted
16	1[illegible]	rulngs	rulings
16	12	table XI	Table X
20	1	squares	square
23	17	only prepared	only slides prepared
2[illegible]	3[illegible]	nomal	normal
26	13	nomal	normal
26	10	cessaion	cessation
29	16	th	the
3[illegible]	3	cell volume	cell volume table
3[illegible]	22	absoult	absolute
34	20	devitation	deviation
37	3	cobalton	cobalt
[illegible]	29	microlytic	macrocytic
48	21	self	itself
	33	Israel	Israel
[illegible]	[illegible]	dsvelop	develop
61	9	pyenotic	pyknotic
67	21	nomal data	normal data (under Table XIV)
7[illegible]	8	p 32	P^{32}
81	21	S	P
86	10	cyringe	syringe
90	11	centrifugilisation	centrifugalisation

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Page	Line	For	Read
91	19	fagility	fragility
92	22	filbrinogen	fibrinogen
93	last line	coton wool	cotton wool
94	2	picking	pricking
96	26	of	oft
99	11	alread	already
100	44	abberation	aberration
118	34	p 112	p 114
118	under Fig 56	standard and bottle	standard bottle
121	20	stillete	stylet
121	28	thet	the